Volume 59(1) pages 1–220
ISSN 0007–1188

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Annual subscription £45.00 (USA & Canada $99. Rest of world $92). (Orders must be accompanied by remittance. Prices correct at time of publication.)

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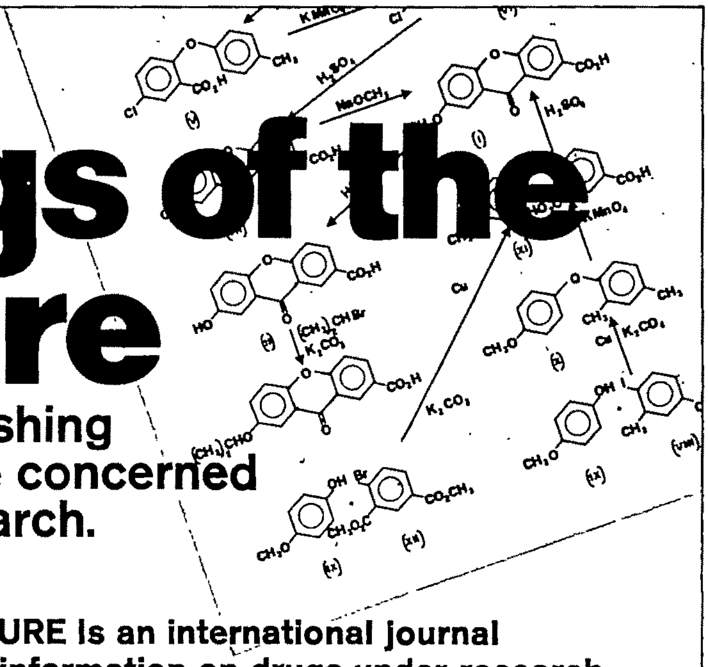

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# EFFECTS OF ADRENOCEPTOR BLOCKADE ON PLASMA CATECHOLAMINE LEVELS DURING ADRENALINE INFUSION

B.J. BRITTON, M.H. IRVING & W.G. WOOD
Surgical Professorial Unit, St. Batholomew's Hospital, London EC1A 7BE

**1** The present experiments investigate the effects of phenoxybenzamine and propranolol, singly and in combination, on plasma catecholamine levels in sheep receiving a three-hour adrenaline infusion.

**2** Five groups of five anaesthetized sheep were studied for a period of 3 h each. One group acted as a control and received only a saline (0.9% w/v NaCl solution) infusion. A second group received a constant infusion of adrenaline (2 µg kg body weight$^{-1}$ min$^{-1}$). A third group received a similar adrenaline infusion, having been premedicated with phenoxybenzamine (1 mg/kg body weight). A fourth group received a similar adrenaline infusion following premedication with (±)-propranolol hydrochloride (7 µg/kg body weight). The fifth group received the adrenaline infusion following premedication with both the $\alpha$- and $\beta$-blocker in the above doses.

**3** Plasma catecholamines were measured on blood samples taken at seven intervals before, during and following the infusion.

**4** Control animals receiving only a saline infusion remained physiologically and biochemically stable throughout the experimental period.

**5** Adrenaline infusion in animals not receiving adrenoceptor blocking drugs caused a rise in plasma adrenaline levels from a low basal value of 1 µg/litre to a maximum level of 19.8 µg/litre. Animals premedicated with phenoxybenzamine exhibited a similar response.

**6** Animals premedicated with propranolol before the infusion of adrenaline did not demonstrate as marked a rise of plasma adrenaline levels as the two previous groups. The maximum mean plasma adrenaline level recorded in this group was 6.88 µg/litre.

**7** Animals premedicated with phenoxybenzamine and propranolol before the infusion of adrenaline showed only a small rise in plasma adrenaline levels compared with animals receiving adrenaline infusion alone. The maximum mean plasma adrenaline level in the group was only 3.43 µg/litre.

**8** The studies demonstrate that by an unknown mechanism $\beta$-adrenoceptor blockade with (±)-propranolol, either alone or in combination with phenoxybenzamine, lowers the plasma adrenaline response evoked by adrenaline infusion.

## Introduction

Adrenaline infusion is frequently used in stress experiments as an agonist to simulate the adrenergic response seen in conditions such as haemorrhagic shock (Halmagyi, Irving & Varga, 1968). Investigation of the effects of adrenaline infusion is facilitated by the use of $\alpha$- and $\beta$-adrenoceptor blocking drugs. It has always been assumed that these drugs do not affect circulating levels of adrenaline and noradrenaline and only exert their effects by receptor blockade. However, Halmagyi, Kennedy & Varga (1971), using haemorrhage as a stress, have demonstrated that certain combinations of adrenoceptor blockade significantly lower the adrenergic response to blood loss as shown by reduced plasma catecholamine levels in the blocked animals. The present experiments investigate the effects of phenoxybenzamine and propranolol, both singly and in combination, on plasma catecholamine levels in sheep receiving a 3-h adrenaline infusion.

## Methods

Twenty-five female Sussex Border crossbreed sheep of mean weight 60.5 kg were used. Prior to the

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experiments they were sedated with ketamine (Ketalar, Parke-Davis) 20 mg/kg body wt. intramuscularly. This facilitated transport and positioning. Thirty minutes later, anaesthesia was induced with thiopentone (500 mg i.v.), and maintained with intermittent intravenous chloralose, 1% in 0.9% w/v NaCl solution (saline). The trachea was intubated with a cuffed Magill tube and the animals allowed to breathe spontaneously.

A plastic sampling cannula (6 F.G. Portex) was inserted into the left femoral vein and advanced into the vena cava to allow sampling of central venous blood. Both antecubital veins were cannulated with fine plastic catheters to allow intravenous infusions of adrenaline, saline and adrenoceptor blocking drugs. Infusions were given by constant volume pumps (Delta, Watson-Marlow) delivering 1 ml/minute. A mercury manometer was connected to a plastic cannula in the right common carotid artery to measure mean arterial pressure.

The animals were allowed to settle for 30 min after the initial surgical procedure before a resting blood sample was taken. By this time the effects of the ketamine had worn off (Baraka, Harrison & Kachachi, 1973).

Five groups of five sheep were investigated, the order of medication being allocated from random number tables. The treatment for the groups was as in Table 1.

Adrenaline was given as adrenaline bitartrate, phenoxybenzamine as phenoxybenzamine hydrochloride (Dibenyline, Smith, Kline & French) and propranolol as (±)-propranolol hydrochloride (Inderal, I.C.I.). All solutions were made up in saline.

Blood samples were taken as follows:

1. Resting control.
2. Resting control after administration of adrenoceptor blocking drugs, where given.
3. Thirty minutes after start of adrenaline infusion, where given.

4–6. At 60, 120 and 180 min after start of adrenaline infusion.

7. Sixty minutes after cessation of 3 h adrenaline infusion, where given.

Blood samples (10 ml) were taken from the vena cava into lithium heparin tubes containing 5 mg sodium metabisulphite as antioxidant (Carruthers, Taggart, Conway, Bates & Somerville, 1970). The blood was centrifuged immediately and the plasma transferred to plastic tubes which were placed on dry ice. Plasma samples were kept at −76°C until assay, within 7 days.

Catecholamines were measured according to the semi-automated method of McCullough (1968), which measures noradrenaline and total catecholamines, adrenaline being determined by difference. Cross reaction of adrenaline in the noradrenaline assay is less than 2%, and interference from catecholamine metabolites is low. The only interference from the catecholamine-like group of compounds in use in medical practice comes from isoprenaline and $\alpha$-methylnoradrenaline (formed by the $\beta$-hydroxylation of $\alpha$-methyldopa) neither of which was used in this study.

## Results

Changes in plasma catecholamine levels in each group were compared with the levels in period 2. This period was chosen as representing the basal value after administration of the adrenoceptor blocking drugs, where given.

In the groups receiving phenoxybenzamine (Group C), propranolol (Group D), and phenoxybenzamine + propranolol (Group E), additional comparisons were made with the corresponding period in the animals given an adrenaline infusion without blockers (Group B). Plasma catecholamine levels are expressed throughout as µg/litre, and figures quoted unless otherwise stated are mean (± standard deviation). Intragroup comparisons have been made using paired *t*-test, and intergroup comparisons by two-sample *t*-test.

### *Group A. Control (Tables 2 & 3, Figure 1)*

The results in this group of animals demonstrated that the anaesthetized animal lying supine and being

**Table 1** Infusion schedules

| *Group* | *Pump 1* | *Pump 2* |
|---|---|---|
| A | Saline | Saline |
| B | Adrenaline[1] | Saline |
| C | Adrenaline[1] | Phenoxybenzamine[2] |
| D | Adrenaline[1] | Propranolol[3] |
| E | Adrenaline[1] | Phenoxybenzamine[2] + propranolol[3] |

[1] 2 µg body wt.$^{-1}$ min$^{-1}$; [2] 1 mg/kg body wt. in saline over 15 min given 45 min before the infusion of adrenaline, then saline; [3] 7 µg/kg body wt. in saline. Continuous infusion from 45 min before infusion of adrenaline.

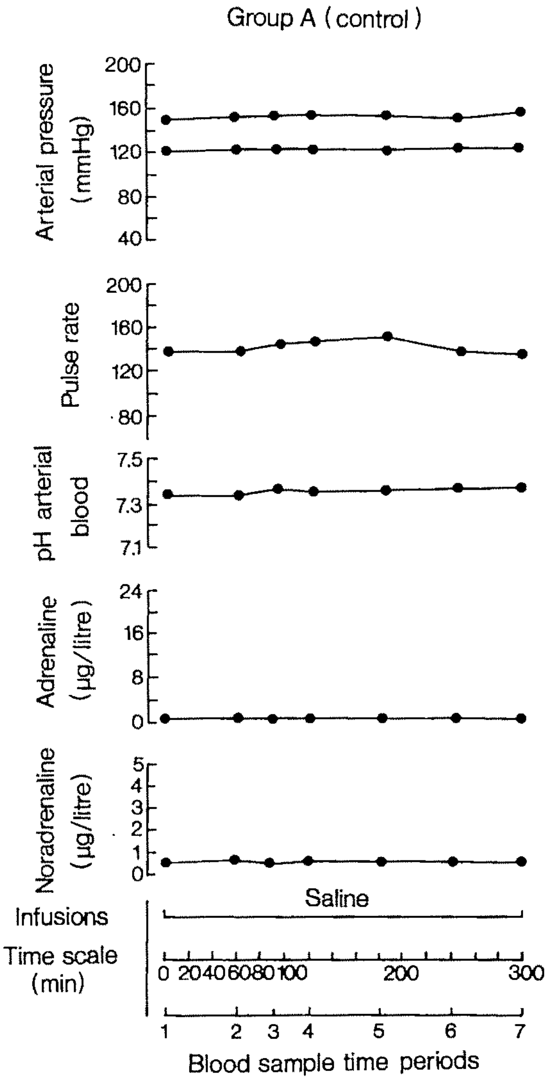

**Figure 1** Pulse, blood pressure, adrenaline and noradrenaline concentrations in saline-infused sheep, Group A (control). Mean values.

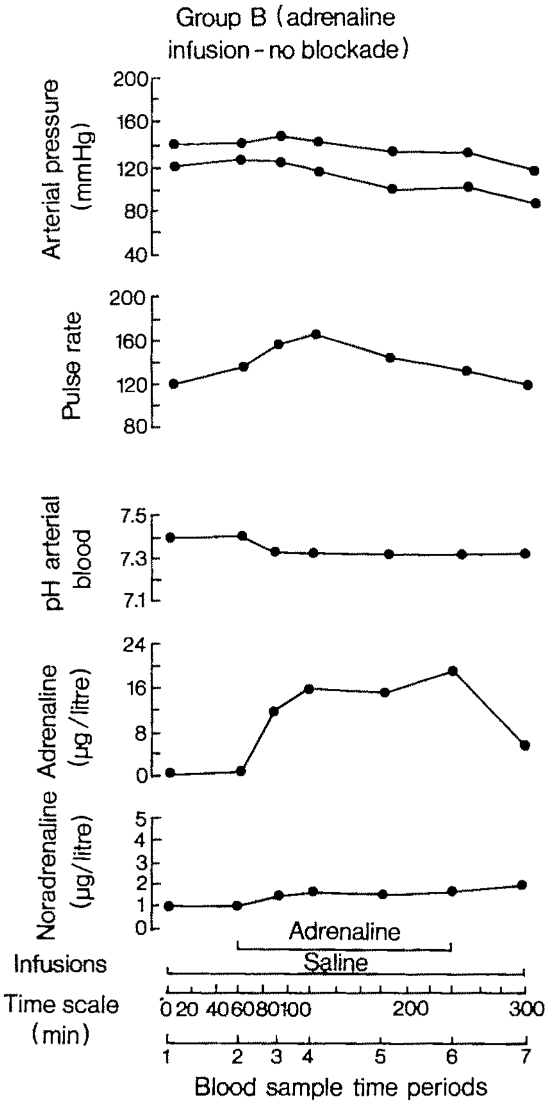

**Figure 2** Pulse, blood pressure, adrenaline and noradrenaline concentrations in sheep infused with adrenaline alone, Group B. Mean values.

infused with saline is physiologically and biochemically stable throughout the experimental period. The maximum adrenaline level recorded in this group was in period 3, 0.92 (±0.37), and the maximum noradrenaline level in period 2, 0.75 (±0.13). Throughout the experiment pulse rate, arterial blood pressure and pH remained stable.

### *Group B. Adrenaline infusion (Tables 2 & 3, Figure 2)*

In this group, the physiological response to adrenaline infusion was similar to that described previously. There was an increase in pulse rate and a gradual fall in arterial pressure (Irving, Britton, Wood, Padgham & Carruthers, 1968). Infusion of adrenaline caused a rise in plasma adrenaline levels from a low basal value of 1.00 (±0.58) to maximum levels in period 6 of 19.8 (±9.27). The adrenaline levels in periods 3–7 were all significantly raised from period 2. (3. $P<0.02$; 4. $P=0.01$; 5. $P<0.01$; 6. $P>0.01$; 7. $P<0.02$). There were no significant changes in the plasma noradrenaline levels throughout the observation period.

*Group C. Phenoxybenzamine plus adrenaline (Tables 2 & 3, Figure 3)*

In these animals, phenoxybenzamine premedication caused a fall in arterial pressure which became more marked after the start of the adrenaline infusion. The rise in plasma levels of adrenaline during the course of the experiment showed similar trends to Group B. Maximum levels of adrenaline were seen in period 5 of 21.56 (±15.36) and plasma adrenaline was significantly raised above period 2 in periods 3–6 (3. $P<0.01$; 4. $P<0.01$; 5. $P<0.01$; 6. $P<0.05$). Noradrenaline levels were significantly higher in period 7 ($P>0.02$). At no time were adrenaline or noradrenaline levels different from Group B.

*Group D. Propranolol plus adrenaline (Tables 2 & 3, Figure 4)*

Propranolol prevented the tachycardia which accompanies adrenaline infusion. An initial hypertensive response to the adrenaline infusion was soon followed by a gradual fall in arterial blood pressure. Plasma adrenaline levels during the infusion of adrenaline again rose, but were only significant in periods 5–7 when compared with period 2 (5. $P>0.01$; 6. $P>0.02$; 7. $P=0.05$). The maximum plasma adrenaline level recorded in this group was 6.88 (±2.47). Plasma adrenaline levels were significantly lower than in corresponding periods in the adrenaline infusion group (Group B) in periods

**Table 2** Adrenaline concentration (µg/litres) in central venous blood. Mean ± s.d. during the 7 sampling periods in the saline infusion group (A), adrenaline infusion group (B), phenoxybenzamine premedicated group (C), propranolol premedicated group (D), and phenoxybenzamine and propranolol premedicated group (E).

| *Group* | *Period 1* | *2* | *3* | *4* | *5* | *6* | *7* |
|---|---|---|---|---|---|---|---|
| A | 0.91<br>±0.36 | 0.81<br>±0.09 | 0.92<br>±0.37 | 0.66<br>±0.25 | 0.74<br>±0.41 | 0.52<br>±0.33 | 0.65<br>±0.24 |
| B | 0.57<br>±0.44 | 0.67<br>±0.43 | 12.80<br>±5.96 | 16.40<br>±6.82 | 15.60<br>±5.93 | 19.80<br>±9.27 | 6.55<br>±2.97 |
| C | 1.30<br>±1.42 | 1.44<br>±1.31 | 10.20<br>±4.13 | 18.50<br>±17.90 | 21.60<br>±15.40 | 14.20<br>±10.60 | 9.99<br>±7.47 |
| D | 0.85<br>±0.63 | 0.74<br>±0.34 | 5.96<br>±1.38 | 5.72<br>±2.32 | 6.88<br>±2.47 | 6.79<br>±5.40 | 2.96<br>±2.21 |
| E | 0.38<br>±0.23 | 1.04<br>±0.87 | 3.43<br>±2.48 | 3.31<br>±2.54 | 2.11<br>±0.83 | 1.78<br>±0.92 | 2.66<br>±2.71 |

**Table 3** Noradrenaline concentration (µg/litre) in central venous blood. Mean ± s.d. during the 7 sampling periods in the saline infusion group (A), adrenaline infusion group (B), phenoxybenzamine premedicated group (C), propranolol premedicated group (D), and phenoxybenzamine and propranolol premedicated group (E)

| *Group* | *Period 1* | *2* | *3* | *4* | *5* | *6* | *7* |
|---|---|---|---|---|---|---|---|
| A | 0.68<br>±0.11 | 0.75<br>±0.13 | 0.57<br>±0.30 | 0.68<br>±0.30 | 0.60<br>±0.28 | 0.68<br>±0.39 | 0.71<br>±0.25 |
| B | 0.98<br>±0.54 | 1.00<br>±0.58 | 1.28<br>±0.67 | 1.50<br>±0.72 | 1.23<br>±0.51 | 1.83<br>±0.83 | 2.39<br>±2.07 |
| C | 0.70<br>±0.29 | 1.00<br>±0.48 | 1.20<br>±1.09 | 1.71<br>±1.30 | 1.06<br>±0.44 | 0.96<br>±0.40 | 2.44<br>±1.49 |
| D | 1.03<br>±0.59 | 1.27<br>±0.59 | 1.68<br>±1.65 | 1.39<br>±0.79 | 1.41<br>±0.59 | 1.91<br>±1.28 | 2.38<br>±1.32 |
| E | 0.85<br>±0.16 | 0.86<br>±0.19 | 0.58<br>±0.19 | 0.93<br>±0.88 | 0.85<br>±0.32 | 0.80<br>±0.16 | 0.95<br>±0.44 |

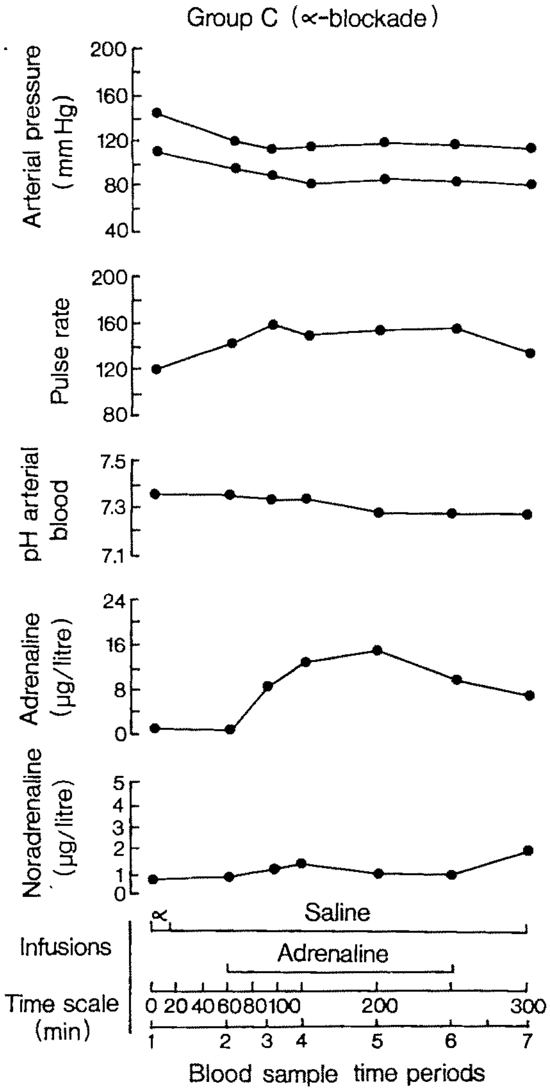

**Figure 3** Pulse, blood pressure, adrenaline and noradrenaline concentrations in sheep infused with adrenaline after pretreatment with phenoxybenzamine, Group C. Mean values.

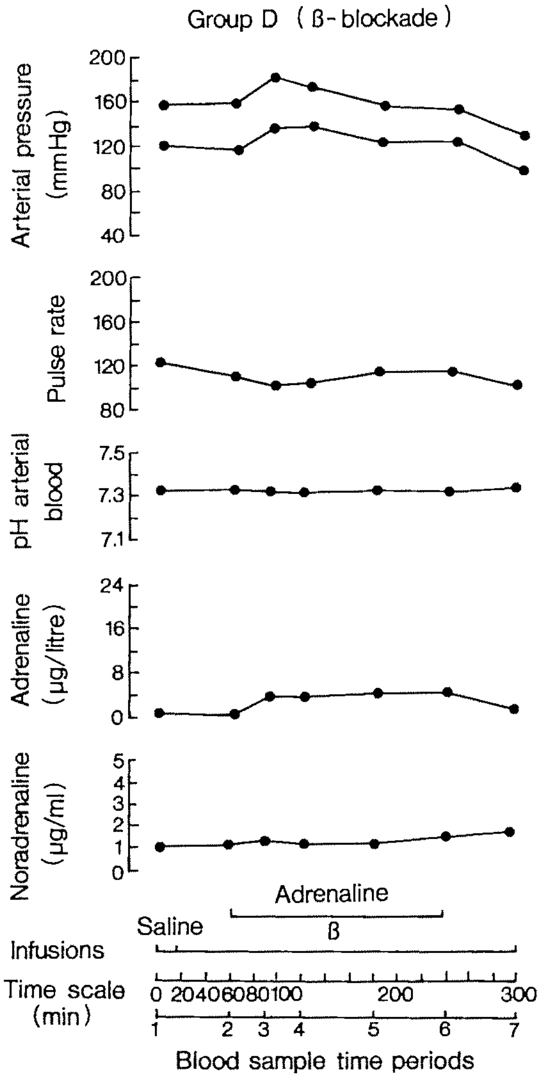

**Figure 4** Pulse, blood pressure, adrenaline and noradrenaline concentrations in sheep infused with adrenaline after pretreatment with propranolol, Group D. Mean values.

3–6 (3. $P<0.05$; 4. $P>0.02$; 5. $P>0.02$; 6. $P<0.05$). Noradrenaline failed to show any significant changes either when compared with period 2 or when compared with Group B.

### *Group E. Phenoxybenzamine plus propranolol plus adrenaline (Tables 2 & 3, Figure 5)*

Combined adrenoceptor blockade produced a stable physiological model in which adrenaline infusion caused little change in pulse rate or pH. There was a slight fall in arterial pressure. The rise in plasma adrenaline levels during adrenaline infusion was small when compared with the animals receiving adrenaline infusion alone (Group B), or after phenoxybenzamine administration (Group C). The maximum plasma adrenaline levels were in period 3, 3.43 (±2.48) and were significantly higher than in period 2 in periods 3–6 (3. $P<0.05$; 4. $P=0.05$; 5. $P>0.01$; 6. $P=0.01$). When compared with the animals receiving adrenaline

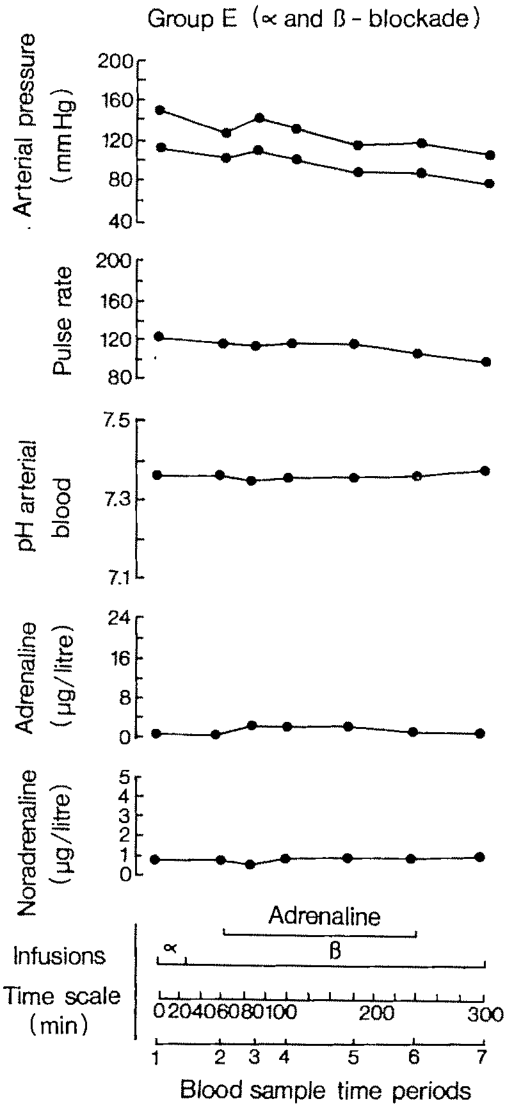

**Figure 5** Pulse, blood pressure, adrenaline and noradrenaline concentrations in sheep infused with adrenaline after phenoxybenzamine and propranolol pretreatment, Group E. Mean values.

infusion alone (Group B), the levels were significantly lower in periods 3–6 (3. $P>0.02$; 4. $P=0.02$; 5. $P=0.01$; 6. $P>0.01$).

In periods 5 & 6, adrenaline levels were significantly lower than in the corresponding period in the animals receiving propranolol (Group D) (5. $P<0.01$; 6. $P=0.05$). Again there were no significant changes in noradrenaline levels, either within or between this group and the others.

## Discussion

Infusion of adrenaline or other sympathomimetic amines has been widely used to investigate their physiological and biochemical effects and to assess the influence of adrenergic antagonists. It has been assumed in most of these investigations that the circulating concentrations of catecholamines are the same in both the control and the adrenoceptor blockade experiments. Recent studies have cast doubt on the accuracy of this assumption (Irving *et al.*, 1974).

The present studies clearly demonstrate that $\beta$-adrenoceptor blockade either alone or in combination with $\alpha$-blockade decreases the rise in plasma adrenaline produced by adrenaline infusion. This observation does not appear to have been made before, although Halmagyi *et al.* (1971) noticed that dogs premedicated with phenoxybenzamine and propranolol prior to haemorrhage did not produce the high levels of adrenaline and noradrenaline observable in non-medicated dogs with an equivalent degree of blood loss and hypotension. However, in their studies, $\beta$-blockade alone did not show a similar effect.

Previous studies have shown that combined adrenoceptor blockage improves the tolerance of sheep and dogs to severe haemorrhagic hypotension and prolonged adrenaline infusion (Halmagyi *et al.*, 1968; Halmagyi *et al.*, 1971). It is known that sustained high circulating levels of catecholamines are deleterious and it may be that the beneficial effect of combined adrenoceptor-blockade results not from receptor blockade but from the lowering of the circulating catecholamine concentrations produced by the drugs.

The explanation for the differences in adrenaline concentration between the four groups of animals in our study is not at the present time clear. Fundamentally, three processes might be altered by adrenoceptor blockade, namely, endogenous secretion of catecholamines in response to adrenaline infusion, uptake of catecholamines into tissues and, thirdly, enzymatic breakdown of the hormones.

It is known that infusion of sympathomimetic amines induces an additional endogenous release of adrenaline from the adrenal gland (Walker, Zileli, Reutter, Shoemaker, Friend & Moore, 1959). However, it would seem unlikely that adrenoceptor blockade, either $\alpha$- or $\beta$-, would influence this release because the neurogenic synapse within the adrenal medulla is cholinergic.

Uptake mechanisms are the principal means for the inactivation of catecholamines *in vivo*. There are two types of uptake. The first is the return of catecholamines to the sympathetic neurone from which they were released, known as Uptake$_1$. The second is Uptake$_2$ where the catecholamines are taken up into other cells; such as smooth muscle. Uptake$_1$ is saturated at low external catecholamine concentrations and shows a preference for noradrenaline. $\text{Uptake}_2$ predominates at high external catecholamine concentrations and has a higher affinity for adrenaline than for noradrenaline. The capacity of $\text{Uptake}_1$ is low whilst that of $\text{Uptake}_2$ is high (Lightman & Iversen, 1969).

Phenoxybenzamine is known to inhibit both $\text{Uptake}_1$ and $\text{Uptake}_2$ (Iversen, 1973; Starke, 1973) and it is also known that phenoxybenzamine will increase the stimulation-induced overflow of noradrenaline from nerve endings. Clearly this could mean that in the Group C animals the adrenaline concentrations are increased as a result of increased overflow. However, it would be expected that noradrenaline would be increased as well, which it was not. Also this does not explain the reduced concentrations of adrenaline in Group E.

Propranolol inhibits $\text{Uptake}_1$ but its action on $\text{Uptake}_2$ is unknown (Euler & Lishajko, 1968) although it is known that the closely related pronetholol inhibits $\text{Uptake}_2$ (Eisenfeld, Axelrod & Krakoff, 1967) and that propranolol itself will inhibit noradrenaline uptake into platelets (Bygdeman & Johnsen, 1969). However, in order to reduce the adrenaline concentration by this mechanism it is necessary to postulate that propranolol facilitates uptake even in the presence of phenoxybenzamine. Facilitation of uptake has never been described.

Catabolism of catecholamines occurs as a result of the action of two main enzyme systems. Catechol-*O*-methyltransferase (COMT) occurs principally in the liver and normally selectively methylates the 3-OH group on the catechol ring. COMT converts adrenaline and noradrenaline to their respective metanephrines. Jarrott (1973) has shown that up to 60% of COMT activity remains after sympathectomy showing the majority to be extraneuronal. In considering the effect of adrenoceptor blocking drugs this extraneuronal COMT could play an active part in inactivating excess adrenaline and noradrenaline, particularly as the patterns of blood flow to various organs, including the liver, will be altered by adrenoceptor blockade. Bohuon & Assicot (1973) have extensively studied COMT activity in both liver and erythrocytes. Amongst inhibitors of COMT *in vitro* phenoxybenzamine and propranolol were studied. Membrane bound COMT of erythrocytes was inhibited by low concentrations of propranolol. In erythrocyte ghost fractions a 17% inhibition of COMT was achieved at $10^{-10}$ M, whereas in the solublized membrane fraction 4% inhibition was seen at propranolol concentrations of $10^{-4}$ M. In the soluble fraction of the cell no inhibition by propranol was seen at this concentration. Phenoxybenzamine showed no inhibition of COMT activity. It has also been shown that high circulating catecholamine levels may induce COMT activity reflected in the urinary output of metanephrines after periods of catecholamine activity and infusion during open heart surgery (Hine, Wood, Mainwaring-Burton, Butler & Irving, 1975). As the animals in our study were not catheterized and plasma metanephrine concentration could not be measured, no estimate of COMT activity is available.

The second major enzyme system in the degradation of catecholamines is monoamine oxidase (MAO). This enzyme is not inhibited by $\alpha$- or $\beta$-blockers in the doses used in these experiments.

A third degradation system (see Erwin, 1973) consists of two enzymes, an NAD-dependent aldehyde dehydrogenase and an NADP-dependent aldehyde reductase. These enzymes convert the aldehydes resulting from MAO activity to their corresponding acid or alcohol respectively. It is not known whether they are inhibited by adrenoceptor blocking drugs and they only have an effect after the catecholamines have been inactivated by MAO. In conclusion, therefore, it would seem unlikely that interference with the degradation enzymes for catecholamines would explain our findings.

Finally, these studies demonstrate that investigation of a sympathetic response using $\beta$-blockade may result in different plasma catecholamine levels in the control and the test experiments. Interpretation of the results must therefore be undertaken with care and in the light of the actual catecholamine concentrations.

These studies were supported by British Heart Foundation Grant No. 431. W.G.W. was supported in part by the Wellcome Foundation. Dr P.V. Cole of the Department of Anaesthetics provided technical services. We acknowledge the help of Mr M.J. Butler and Mr R.W. Mainwaring-Burton in the preparation of the manuscript.

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*(Received August 18, 1975.*
*Revised July 9, 1976.)*

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# POTENTIATION OF THE RESPONSE TO VASOPRESSIN (PITRESSIN) BY TREATMENT WITH A COMBINATION OF CHLORPROPAMIDE AND CHLOROTHIAZIDE IN BRATTLEBORO RATS WITH HEREDITARY HYPOTHALAMIC DIABETES INSIPIDUS

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1 The effect of a combination of chlorpropamide and chlorothiazide in Brattleboro rats with hereditary hypothalamic diabetes insipidus (DI) treated with low doses of vasopressin (Pitressin tannate in oil) was investigated with particular reference to the time course of response from the initiation of treatment.

2 Analysis of the relationship between water intake and body weight indicated no real correlation and body weight accounted for only 4.4% of the variation in water intake. It was therefore decided to use whole body responses as the index in preference to the response per unit body weight.

3 The daily administration of 5 mg chlorpropamide combined with chlorothiazide in the drinking water (4 mg/l) to Pitressin-treated DI rats potentiated the response to small doses of vasopressin (25 and 50 mu Pitressin/24 hours). Water intake was reduced by the drug combination by an average of 12.35 ml/24 h, but only on the second day of treatment was the decrease of any real magnitude (30 ml/24 h but otherwise 9 ml/24 h or less). Analysis of urine volume measurements gave similar results to those obtained for water intake and the potency ratio measured in terms of free water clearance was 1.26 (agreeing closely with the ratio for water intake which was 1.24).

4 A reduction in the solute excretion was observed only in those DI rats treated with the higher dose of Pitressin (50 mu/24 h) combined with the two drugs.

5 Possible reasons for the discrepancy between the effect of the combination of chlorpropamide and chlorathiazide on water metabolism in the DI rat and the DI patient are discussed.

## Introduction

In studies on the effect of chlorpropamide in Brattleboro rats with hereditary diabetes insipidus (DI) it has been shown that the drug appears to potentiate the antidiuresis induced by small amounts of exogenous vasopressin (Berndt, Miller, Kettyle & Valtin, 1970; Miller & Moses, 1970). Laycock, Lee & Lewis (1974) recently showed that while the potentiating effect of chlorpropamide did exist in these experimental animals treated with a small amount of vasopressin (25 mu Pitressin/24 h) this effect was minimal and could not be compared with the sometimes dramatic reductions in water excretion observed in many patients with diabetes insipidus (e.g. Arduino, Ferraz & Rodriguez, 1966; Wales & Fraser, 1971).

Chlorothiazide, on the other hand, has been shown to have an antidiuretic effect independent of vasopressin in an uncontrolled trial on patients with DI (Crawford, Kennedy & Hill, 1960), rats with experimental DI (Skadhauge, 1966) and in Brattleboro DI rats (Laycock, 1973). In the present study, the effect of a combination of the two drugs, chlorpropamide and chlorothiazide, has been measured in the Brattleboro DI rat treated with low doses of Pitressin to examine whether the antidiuresis is significantly improved by such a therapy. In addition, the time course of the changes in water intake, urine volume and solute output were studied, in order to determine the possible mechanism (or mechanisms) involved in potentiating the actions of the antidiuretic hormone, vasopressin.

## Methods

The general methodology used for this experiment has been described by Laycock *et al.* (1974). Sixteen female adult Brattleboro DI rats weighing between 175 and 240 g were divided into four equal groups (A, B, C and D). Rats in groups A and B were placed in individual metabolism cages, while those in groups C and D were placed in individual normal plastic cages allowing water intake measurements only.

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A 2 × 2 bioassay was performed on each group of rats simultaneously using a Latin square treatment schedule within each group to balance animal and time effects. Each one of four treatments was given for four days, during which water and/or urine measurements were made daily, and this was followed by an interval of three days before the consecutive treatment of the schedule. (A pilot study had previously shown that this interval was adequate to eliminate residual effects from the various treatments.) The standard drug vasopressin was used in the form of Pitressin tannate in oil; this was injected subcutaneously into the dorsal surface of each rat in doses of 25 and 50 mu/24 h (Treatments S1 and S2). The responses to these doses were known to be submaximal (Laycock & Williams, 1973). Treatments T1 and T2 consisted of the same two doses of Pitressin respectively, combined with the two drugs under investigation. These were chlorpropamide, also injected subcutaneously at a dose of 5 mg/24 h, and chlorothiazide which was added to the drinking water at a concentration of 4 mg/l so that the daily dose to each rat was around 0.5 mg.

This experimental design was therefore completed in four weeks and provided data for a 4 × 4 Latin square which for water intake was replicated four times so that there was information about the effect of housing the animals in metabolism cages. For each animal/treatment combination there were four sequential daily observations and these provided information about any trend during treatment.

## Results

### *Choosing an index of response*

While it is common practice to express water intake or urine output per 100 g body weight, no evidence of a correlation between body weight and these two volumes has been presented in the literature. When no correlation exists, an index of response where water intake or urine output is expressed per 100 g body weight becomes inappropriate for such a derived index must inevitably have a higher error variance (Lewis, 1974). It is therefore necessary to determine a suitable response index for this particular experiment.

The initial and final body weights of the 16 animals used in the experiment together with the total water intake for the 16 days of observation are given in Table 1. Every animal received each one of the four treatments during this period and the relationship between these totals should be little affected by the treatments. The range of the water intake values during the experiment was highest on Treatment S1 at 157.5 ml/24 h and lowest on Treatment T2 at 105.9 ml/24 hours. There was almost zero correlation ($r = 0.007$) between the water intakes and the average of the initial and final body weights for each animal. This point was explored in more detail by using analysis of covariance to determine the correlation

**Table 1** The initial and final body weights of the sixteen DI rats used in the experiment together with the total water intake for each rat over the 16 days of treatment (see text)

| | | *Initial weight* | *Final weight* | *Total water intake (16 days)* |
|---|---|---|---|---|
| Group A | 1 | 225 | 215 | 2199 |
| | 2 | 220 | 217 | 1831 |
| | 3 | 240 | 245 | 2123 |
| | 4 | 240 | 250 | 2343 |
| Group B | 1 | 205 | 220 | 2387 |
| | 2 | 190 | 200 | 1606 |
| | 3 | 175 | 200 | 2124 |
| | 4 | 200 | 210 | 2184 |
| Group C | 1 | 210 | 220 | 2078 |
| | 2 | 235 | 232 | 2118 |
| | 3 | 235 | 245 | 1887 |
| | 4 | 215 | 225 | 2846 |
| Group D | 1 | 225 | 220 | 2065 |
| | 2 | 215 | 210 | 2954 |
| | 3 | 195 | 202 | 889 |
| | 4 | 220 | 242 | 1227 |

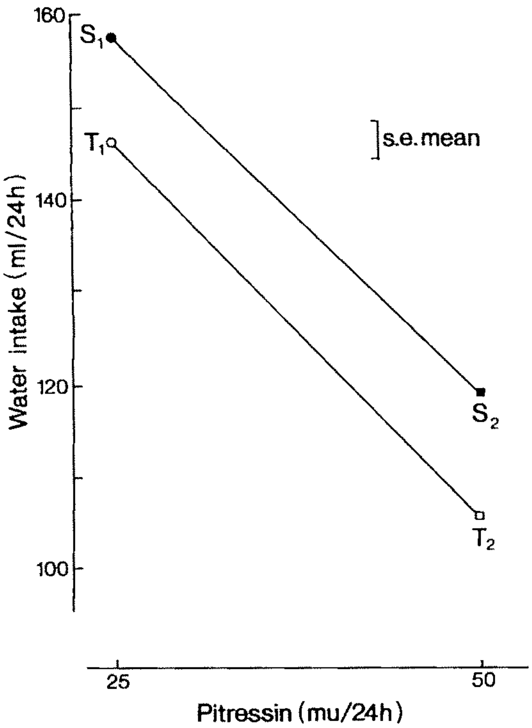

**Figure 1** The 2 x 2 assay for the mean water intake of 16 Brattleboro DI rats treated with 25 and 50 mu Pitressin/24 h (treatments S1 and S2) and with the same two doses of Pitressin/24 h combined with the two drugs chlorpropamide and chlorothiazide (treatments T1 and T2).

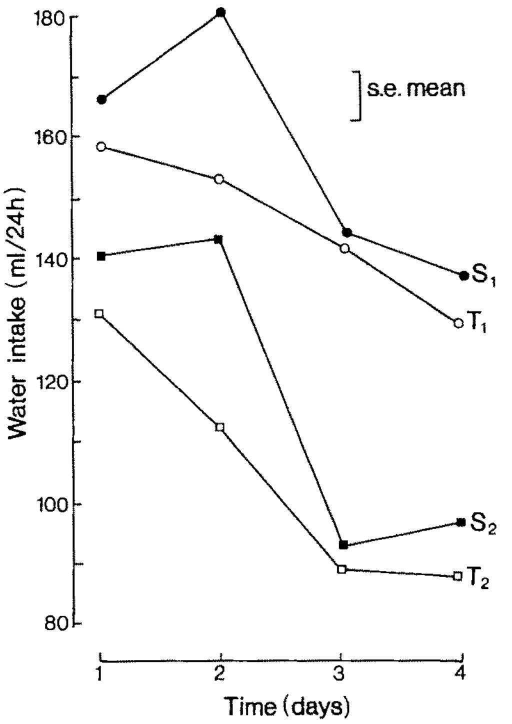

**Figure 2** The mean daily water intake of the 16 Brattleboro DI rats on each of the four days of treatment with 25 and 50 mu Pitressin/24 h (treatments S1 and S2) and the same two doses of Pitressin/24 h combined with the two drugs chlorpropamide and chlorothiazide (treatments T1 and T2).

within groups after adjusting for any differences in treatment response. It was shown that the degree of correlation was still not significant and body weight accounted for only 4.4% of the variation in water intake. It was therefore decided to use whole body responses as the index in preference to the response per unit body weight.

### *The effect of the drug combination on water intake*

Water intake measurements were available for all four groups of animals, and the overall response is shown in Figure 1. This assay satisfied the requirement of similarity (parallelism of the log-dose response lines, $P=0.80$), and the effect of doubling the dose of vasopressin was to decrease water intake by 39.3 ml/24 h ($P<0.001$). The effect of the combination of the two drugs chlorpropamide and chlorothiazide decreased water intake by 12.35 ml/24 h ($P<0.01$), giving a potency ratio of 1.24 (95% confidence limits: 1.06, 1.50). On each day of treatment the drug effect reduced water intake (see Figure 2) but only on the second day was it of any real magnitude (30 ml/24 h but otherwise 9 ml/24 h or less).

### *The effect of the drug combination on urine and solute outputs*

Values for the urine and solute output were available for the eight animals in groups A and B and analysis of the urine output gave similar results to those obtained from water intake measurements (Table 2), the daily urine volume being on average 22.7 ml less than the daily water intake.

The daily solute output was calculated from the known urine volumes and urine osmolalities. The effect of the four treatments (S1, S2, T1 and T2) upon the solute excretion of DI rats is shown in Figure 3. A considerable degree of interaction exists and there is therefore no evidence for similarity between the treatment responses. On the basis of the results, only treatment T2 had any material effect on the solute output, eliminating the increase on day 2 (Figure 3b)

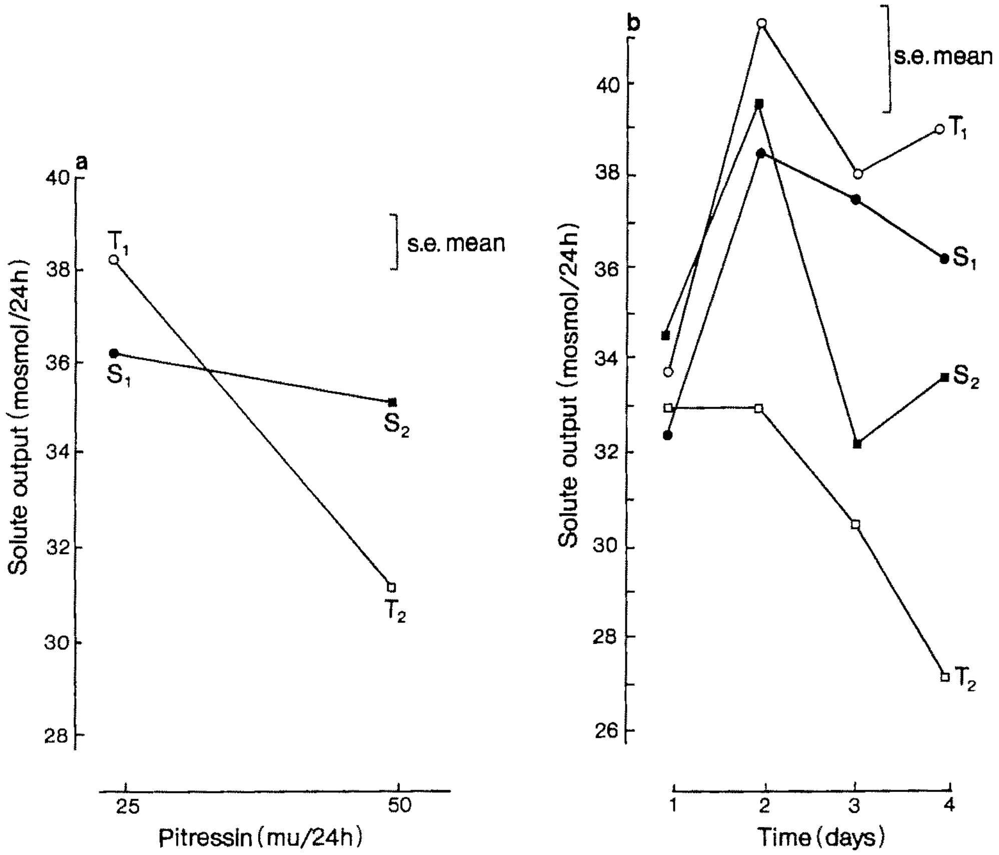

**Figure 3** (a) The 2 × 2 assay for the mean total solute output of eight Brattleboro DI rats treated with the four treatments S1, S2, T1 and T2 (see legend for Figure 1). (b) The mean solute excretion of the same DI rats on each of the four days of treatment with S1, S2, T1 and T2.

and yielding a more progressive reduction in solute excretion over the 4 days (Figure 3a) than the other three treatments.

The relationship between urine volume and solute excretion is shown in Figure 4a. The insertion of an iso-osmotic line with a slope equal to the reciprocal of 0.3 mosmol/kg (assuming a rat plasma osmolality of 300 mosmol/kg) permitted rapid graphical determination of the response in terms of free-water clearance, thus avoiding an otherwise tedious calculation. The vertical distance from the zero free-water clearance line in Figure 4b for the four treatments is equal to the vertical distance from the iso-osmotic line in Figure 4a. The potency ratio for this assay is 1.26.

**Table 2** The mean differences between water intake and urine output values for the eight rats in groups A and B (see text) for the four treatments used in the experiment

| | *Treatment* | | | | |
|---|---|---|---|---|---|
| *Days* | *S1* | *S2* | *T1* | *T2* | *Mean* |
| 1 | 20.88 | 17.25 | 31.38 | 14.75 | 21.09 |
| 2 | 22.63 | 20.63 | 24.13 | 21.88 | 22.31 |
| 3 | 25.25 | 18.25 | 27.88 | 20.13 | 22.88 |
| 4 | 26.62 | 21.63 | 28.50 | 21.75 | 24.63 |
| Mean | 23.84 | 19.44 | 27.97 | 19.66 | 22.73 |

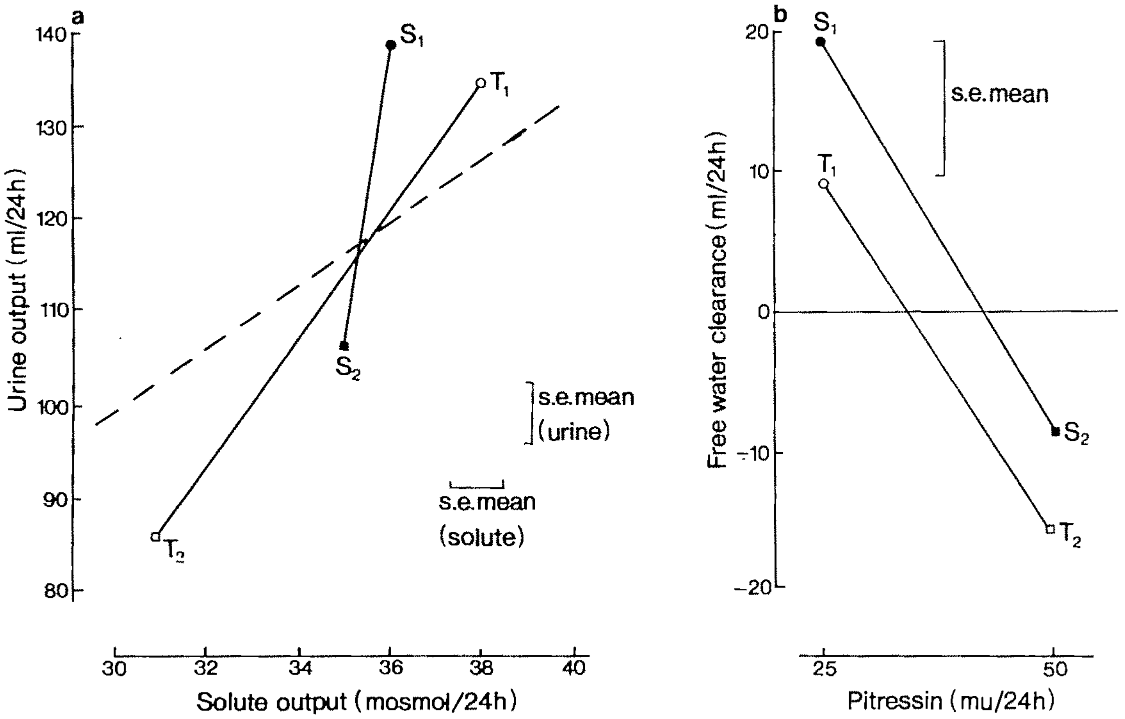

**Figure 4** (a) The relationship between the mean overall urine and solute excretion of eight Brattleboro DI rats with the four treatments S1, S2, T1 and T2 (see legend to Figure 1). The dashed line represents the estimated iso-osmotic line (see text). (b) The overall effect of the four treatments (S1, S2, T1 and T2) on the free-water clearance in the eight DI rats.

## Discussion

In studies upon patients suffering from diabetes insipidus, both chlorpropamide and various thiazides have been shown to exert an antidiuretic effect. Webster & Bain (1970) reported complete control with chlorpropamide alone in six out of eleven patients suffering from either idiopathic diabetes insipidus or a secondary form stemming from a known hypothalamic or pituitary lesion. The remaining five patients were controlled by a combination of chlorpropamide and a thiazide. These authors concluded that chlorpropamide alone excited a powerful effect in controlling the diuresis in vasopressin-deficient patients. Their results also showed an enhancement of the effect of chlorpropamide by the addition of the thiazide (hydrochlorothiazide) although fully controlled studies were not made.

In the present experiments, the effect of a combination of chlorpropamide and chlorothiazide on the water balance of Brattleboro rats with hereditary hypothalamic DI treated with small doses of Pitressin was examined. The 5 mg/24 h dose of chlorpropamide injected into each DI rat in this experiment compared with doses generally administered to DI patients (250–500 mg/24 h) when calculated on a surface-area ratio basis. The quantity of chlorothiazide ingested by each rat (average weight 220 g) daily was of the order of 0.5 mg/24 h, which dose using the same surface-area ratio would correspond to some 20 mg/24 h to an average DI patient weighing some 70 kg. Such a dose of chlorothiazide would be unlikely to have any direct antidiuretic effect in treating a patient with DI, on its own, so that it was hoped that if it also had an antidiuretic effect via the chlorpropamide-Pitressin system, such an effect might be detectable in the present experiment. However, the potentiation of the vasopressin-induced antidiuresis by the drug combination was shown to be significant, but surprisingly small when compared to the clinically relevant effect observed in many DI patients. The administration of the two drugs chlorpropamide and chlorothiazide with 50 mu Pitressin/24 h (treatment T2) no longer produced an increased solute output on any of the four days of treatment but gave a progressive reduction in output over the four days.

A response expressed in terms of free water clearance gives a very similar overall potency ratio (1.26 against 1.24 for water intake) but with slightly more evidence of interaction between the two treatments (shown by the slight convergence of the response lines). On this basis, water intake is the preferred response variable for bioassay.

Another feature of interest concerned the daily trend of the drug-effect, most clearly shown for the water intake measurements (Figure 2). The drug effect was present on all four days of treatment, but only on the second day of treatment was it of real importance. Since the effect due to the vasopressin alone increased to reach a maximum on the third or fourth day of treatment, these time trends could indicate that the combination of drugs might increase the velocity with which the concentrating ability of the kidney is stimulated by the action of vasopressin. One possible mechanism could be to increase the medullary interstitial fluid concentration which is a prerequisite for the maintenance of the countercurrent multiplier loop.

To conclude, the administration of a combination of chlorpropamide and chlorothiazide in the present doses to Brattleboro DI rats treated with small doses of Pitressin is insufficient to restore their water balance to levels which are comparable to those observed in normal rats. This observation differs from the reported extremely successful effect of comparable doses of these drugs in DI patients (e.g. Webster & Bain, 1970). However, there is evidence of a dose-dependent action of vasopressin on solute excretion in these experimental animals which is modified by combination with the other drugs. Since the possibility of a renal defect in the Brattleboro DI rat cannot be discounted, further studies using this experimental model are necessary.

J.F.L. is grateful for the support of the MRC during the completion of this work. The Pitressin tannate in oil was kindly donated by Parke, Davis and Company, and the chlorpropamide by Pfizer Limited.

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*(Received December 1, 1975. Revised August 12, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 17–23

# THE $\beta$-ADRENOCEPTOR OF THE HUMAN LYMPHOCYTE AND HUMAN LUNG PARENCHYMA

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1 The response of the $\beta$-adrenoceptors of human lymphocytes to selective agonists and antagonists has been studied quantitatively by measuring changes in cyclic adenosine-3′,5′-monophosphate (cyclic AMP) levels.

2 The receptor was activated by isoprenaline and by salbutamol, and blocked by propranolol but not by practolol. A similar pattern of response was obtained with fragments of human lung tissue.

3 The mean value for $pA_2$ for propranolol was 8.34 and for practolol was 3.95.

4 These findings indicate that the lymphocyte $\beta$-adrenoceptor is a $\beta_2$-receptor and support the validity of using lymphocytes to study $\beta$-adrenoceptor function in bronchial asthma. It may also be of use in the evaluation of selective $\beta_2$-blocking drugs in man.

## Introduction

There is a growing awareness of the need to study disease mechanisms at a cellular level. Work with isolated organs or tissue slices represent moves in that direction, though many artifacts are inherent in their preparation, and their application to man is very limited. Cellular elements of peripheral blood have therefore a great heuristic appeal since they possess receptors specific to a number of endogenous compounds, and they can be isolated repeatedly and with relative ease, even during periods of severe illness. They therefore provide a useful tool for studying a number of cellular mechanisms. Following the discovery of adenylate cyclase in sonicated leucocytes (Scott, 1970) it has been found that these cells possess both $\alpha$- and $\beta$-adrenoceptors (Logsdon, Middleton & Coffey, 1972), histamine receptors (Bourne, Melmon & Lichtenstein, 1971) and prostaglandin $E_1$ receptors (Bourne & Melmon, 1971). Insulin (Gavin, Mann, Buell & Roth, 1972; Gavin, Roth, Neville, De Meyts & Buell, 1974) and growth hormone receptors (Archer, Gorden, Gavin, Lesniak & Roth, 1973) have also been described.

Szentivanyi, as early as 1962, postulated an inherent defect of the $\beta$-adrenoceptor in atopic diseases, including bronchial asthma (see Szentivanyi, 1968). The early work based on this concept used measurements of metabolic and cardiovascular responses to sympathomimetic agents or exercise in intact subjects (Cookson & Reed, 1963; Lockey, Glennon & Reed, 1967; Inoue, 1967; Middleton & Finke, 1968) and the results appeared to support the hypothesis. However, it is not possible to conduct a pharmacologically 'clean' experiment in intact subjects and it would be impossible to conduct such studies on a serial basis throughout severe asthmatic attacks. There has therefore been considerable enthusiasm for the use of leucocytes, either as a mixed population of cells or as purified lymphocytes, since the technique was first suggested by Smith & Parker (1970). Several studies (Logsdon *et al.*, 1972; Parker & Smith, 1972; Alston, Patel & Kerr, 1974; Gillespie, Valentine & Lichtenstein, 1974) have been reported. These authors have all assumed that the $\beta$-receptor of the leucocyte is identical with that in the lung. Before further effort is expended on this model, it is desirable to establish some measure of identity between the two receptors in terms of Land's classification (Lands, Arnold, McAuliff, Luduena & Brown, 1967) into $\beta_1$ (e.g. myocardial) and $\beta_2$ (e.g. bronchial and vascular smooth muscle) receptors. This paper assesses the lymphocyte $\beta$-receptor in terms of its responsiveness to the non-selective and $\beta_2$-selective agonists, isoprenaline and salbutamol respectively. Antagonism of isoprenaline with a non-selective and a $\beta_1$-selective adrenoceptor blocking drug (propranolol and practolol respectively) has also been studied. In addition, preliminary data on the responsiveness of the $\beta$-adrenoceptors in lung parenchyma are presented.

## Methods

Pharmacological response was quantitated by measuring changes in the tissue levels of cyclic-3′,5′-adenosine monophosphate (cyclic AMP). A range of agonist concentrations has been used in the lymphocyte studies to provide complete dose-response curves. A range of concentrations of propranolol and practolol has also been used so that the $\beta$-receptor could be characterized in terms of the $pA_2$ values for these antagonists (Schild, 1947; Arunlakshana & Schild, 1959). No attempt has been made to determine a $pA_2$ in peripheral lung tissue as many different cell types are present, including mast cells and bronchiolar and vascular smooth muscle cells, which may possess different types of $\beta$-receptor.

### *Cell separation*

Lymphocytes were prepared from heparinized venous blood by a modification (Harris & Ukaejiofo, 1970) of Böyum's (1968) technique which produced a cell population of approximately 90–95% lymphocytes. The majority of the remaining cells were monocytes with very few granulocytes. Blood (60 ml) was centrifuged at 250 *g* for 20 min at 15°C (MSE Mistral 4L refrigerated centrifuge), to permit removal of platelet-rich plasma. This was replaced with an equivalent volume of Hanks Balanced Salt Solution (HBSS, Burroughs Wellcome Ltd) previously gassed with 95% $O_2$ and 5% $CO_2$ and adjusted to pH 7.4. Twelve ml aliquots of this 'blood' were carefully layered onto 10 ml Lymphoprep (Nyegaard & Co) and then centrifuged at 400 *g* for 25 min at 15°C. The lymphocytes accumulated at the interface between the two liquids. This layer was harvested, diluted with phosphate buffered saline (Dubelcco's, Oxoid Ltd) at pH 7.4, and then centrifuged at 1000 *g* for 20 min at 15°C. The supernatant was discarded and the cell pellet was gently resuspended in HBSS. The addition of 10% foetal calf serum (Burroughs Wellcome Ltd) to all the media in order to reduce lymphocyte adhesiveness was essential to ensure an adequate yield of cells. Cells were counted in a Coulter Cell Counter. Cell concentrations of $1 \times 10^6$ to $3 \times 10^6$ cells/ml produced a linear response with respect to cell numbers, so the suspension was diluted to give a final concentration of $2 \times 10^6$ cells/ml when all drug solutions had been added. Bovine serum albumin was added to give a final concentration of 0.3 mg/ml and the suspension was buffered with Tris HCl (5 mmol/litre).

### *Human lung*

Tissue was obtained from a patient undergoing lobectomy for bronchiectasis. Normal lung tissue was separated from the diseased area, dissected free of visible bronchi and pleura, and chopped into small pieces. Fragments amounting to 200 mg wet weight were added to each tube and thereafter treated in the same way as lymphocytes except that no protein was added to the incubation mixture. Response was expressed as pmol cyclic AMP per mg protein, the protein being estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

### *Drugs*

The final concentrations of the drugs used (and their sources) were as follows: theophylline $10^{-2}$ mol/l (BDH Ltd); (±)-isoprenaline sulphate $10^{-8}$ to $10^{-4}$ mol/l (Sigma Ltd); (±)-salbutamol sulphate $10^{-8}$ to $10^{-4}$ mol/l (a gift from Allen & Hanbury Ltd); (±)-propranolol hydrochloride $10^{-9}$ to $10^{-4}$ mol/l and (±)-practolol $5 \times 10^{-5}$ mol/l (both gifts from ICI Ltd).

Ascorbic acid (1 mg/ml in stock solutions) was used as an antioxidant for isoprenaline, and was also added to salbutamol for the sake of consistency.

### *Cell incubation*

The incubations were performed in a final volume of 2 ml. To each tube 1 ml of Hanks BSS containing theophylline ($2 \times 10^{-2}$ mol/l) was added, and the tube was placed in a water bath at 37°C. The drugs were then added in volumes of 0.1 ml, the concentrations of each being such that when diluted to 2 ml, the required final concentrations (see above) would be achieved. Finally 1 ml lymphocytes ($4 \times 10^6$ cells/ml) was gently added to each tube, thereby giving a final cell concentration of $2 \times 10^6$ ml. The tubes were gently shaken after the addition and from time to time during incubation to ensure uniform dispersal of the lymphocytes. The incubation was terminated after 15 min by plunging the tubes into boiling water for 5 minutes. Following this, 0.18 pmol [$^3$H]-cyclic AMP (5000 ct/min, New England Nuclear Corp) was added to permit estimation of recovery after purification. The cells were homogenized and centrifuged, and the supernatant applied to $0.5 \times 2.0$ cm columns of Dowex AG $1 \times 8$ (formate form, 200 to 400 mesh, Biorad Labs). The columns were eluted with 10 ml water, which was discarded. Cyclic AMP was then washed from the columns with 12 ml 2 N formic acid which did not elute cyclic-3',5'-guanosine monophosphate (cyclic GMP). The eluate was lyophilized, and the residue was dissolved in 1 ml phosphate-citrate buffer (pH 6.5). Recovery was estimated by counting a 50 µl aliquot of this solution, and further aliquots were used in the cyclic AMP assay described below.

### *Cyclic AMP assay*

A protein binding assay was chosen in preference to a method which depends on measuring the conversion of [$^3$H]-adenosine to [$^3$H]-cyclic AMP, since the latter gives no data on basal cyclic AMP levels.

The method was modified from that of Gilman (1970). The binding protein was extracted from rabbit skeletal muscle, using a protamine-sepharose affinity

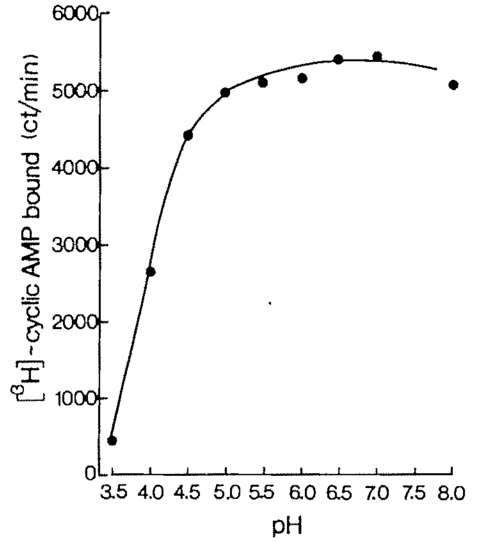

**Figure 1** Binding of cyclic AMP by protein kinase from rabbit skeletal muscle over a pH range of 3.5 to 8.0.

chromatography column (Johnson & Schultz, personal communication). The binding protein was eluted with a 0–2 molar gradient of NaCl, dialysed, and after the addition of bovine serum albumin (1 mg/ml), frozen in 1 ml aliquots at –20°C. Under these conditions it showed no loss of activity for more than 2 years. This protein differed from that described by Gilman in that its ability to bind cyclic AMP was very pH-sensitive, being maximal over a range of pH 5.5 to 7.5 (Figure 1). All assays were performed at pH 6.5.

Each assay tube contained 50 μl phosphate-citrate buffer (pH 6.5) containing known amounts of unlabelled cyclic AMP (0 to 10 pmol) or the experimental material derived from the incubations. To each tube was added 0.5 pmol [³H]-cyclic AMP in 10 μl water (10 μl water alone added to blank tubes), and 50 μl of the binding protein (which had been further diluted with bovine serum albumin). The contents were thoroughly mixed, and allowed to stand in ice for 3 hours. At the end of this time, the incubation mixture was passed through a millipore filter (Millipore HAWP 02400 filter; 0.45 μm pore size) which was then rinsed with ice cold 20 mmolar phosphate buffer (pH 6.0). The filter was dissolved in 1 ml 2-ethoxyethanol in a counting vial, and after the addition of 10 ml Instagel (Packard), the samples were counted in a Packard 3375 spectrometer. Each assay measurement was performed in duplicate. As counting efficiency was very similar for all samples, no correction was made for quenching.

The calibration curve generated at each assay was linear over the range 0.5 to 10.0 pmol. The sensitivity of the assay permitted measurement of cyclic AMP levels as low as 0.1 pmol per assay tube. With known amounts of unlabelled cyclic AMP, the assay was found to be accurate to within ±10%. Other nucleotides were tested for their ability to interfere with this assay. Adenosine triphosphate and adenosine diphosphate were without effect. Cyclic GMP in concentrations 100 times higher than cyclic AMP did interfere with binding, but since it was separated from cyclic AMP by the chromatographic purification the resultant assay may be regarded as specific for cyclic AMP. Estimation of cyclic AMP levels from the calibration curve, and all other calculations involved in this assay were performed on a Digital PDP8/I computer.

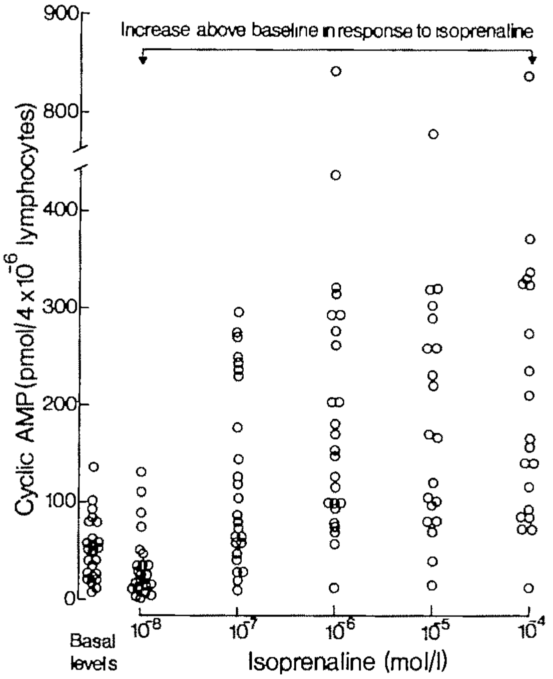

**Figure 2** Basal level of cyclic AMP (pmol per $4 \times 10^6$ lymphocytes) and increase above this level in response to a 15 min incubation with isoprenaline ($10^{-8}$ to $10^{-4}$ mol/l) at 37°C. Observations made on 8 normal subjects studied on 1–5 separate occasions.

## Results

Responsiveness to isoprenaline varied widely both within and between subjects. Figure 2 shows the basal levels of cyclic AMP and the increases seen at each isoprenaline concentration used. The data are derived from 8 normal subjects studied on 24 separate

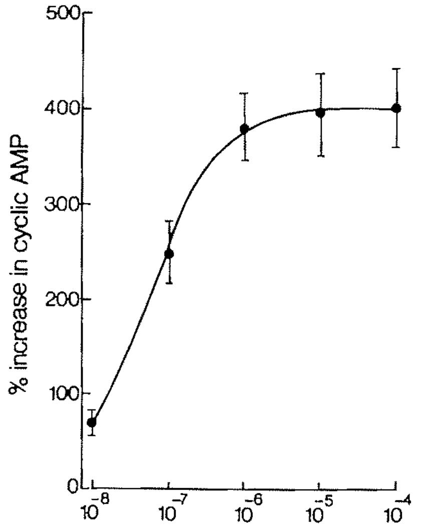

**Figure 3** Percentage increase in cyclic AMP above baseline values in lymphocytes from 8 normal subjects in response to isoprenaline (same studies as shown in Figure 2). Mean values are plotted; vertical lines show s.e. mean.

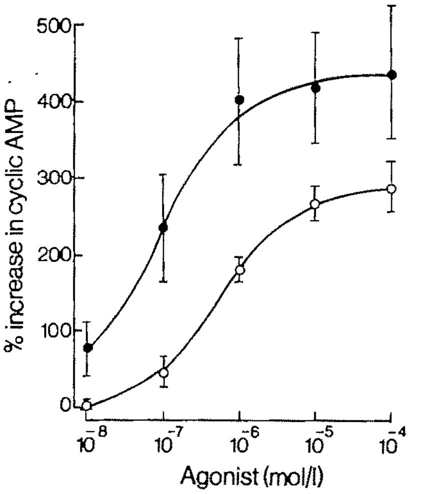

**Figure 4** Percentage increase in cyclic AMP in lymphocytes from 2 normal subjects after 15 min incubation at 37°C with isoprenaline (●) or salbutamol (O).

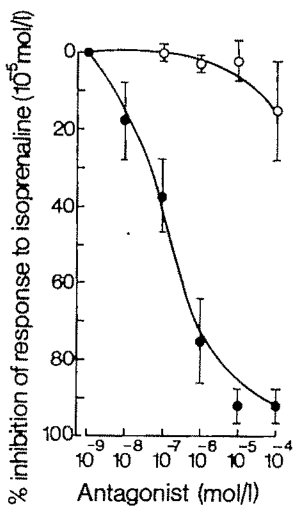

**Figure 5** Antagonism of the increase in cyclic AMP produced by isoprenaline (10$^{-5}$ mol/l) produced by propranolol (●) and practolol (O) in the concentrations shown.

occasions. (In some studies there were insufficient cells to examine the response to every dose in the range employed.) Cyclic AMP levels (expressed as pmol (±s.e.) per $4 \times 10^6$ cells) increased from $48 \pm 5.3$ to $232 \times 25.9$, in the presence of isoprenaline $10^{-4}$ mol/l, an increase of $388 \pm 41.8$%. The cells with the lowest basal levels of cyclic AMP also showed the lowest increases, and *vice versa*, so the data have been analysed in terms of percentage changes, and the resulting dose-response curve for these 8 subjects is shown in Figure 3.

The response to lymphocytes from 2 normal subjects to salbutamol over the same range of concentrations has been examined (Figure 4). This drug produces a dose-response curve with a similar configuration to that of isoprenaline obtained in the same two subjects. Comparison of the two drugs where the curves are roughly parallel indicates that isoprenaline is about 9–15 times more potent, dose for dose, than salbutamol. In addition, salbutamol has only partial agonist activity, producing only a $295 \pm 26$% increase in cyclic AMP as compared with $428 \pm 66$% for isoprenaline.

The comparison between a non-selective (propranolol) and a selective β-blocking drug (practolol) is shown in Figure 5. These data represent observations in 4 normal subjects. The effects of isoprenaline on cyclic AMP levels were progressively antagonized by propranolol, whereas practolol was without effect below $10^{-5}$ mol/litre. No valid dose-ratio can be derived from these data but at a concentration where practolol is beginning to produce some β-blockade ($5 \times 10^{-5}$ mol/l) a potency ratio in the region of 5000 : 1 is apparent.

In order to characterize the receptors further, $pA_2$ values for propranolol and practolol have been derived from Schild plots (Schild, 1947) using cells from 3 normal subjects, and also lymphocytes from a patient with chronic lymphatic leukaemia (Figure 6a,b). The

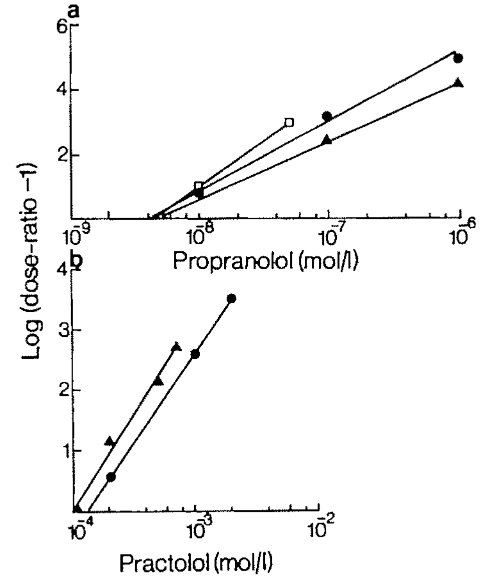

**Figure 6** (a) Schild plot showing the inhibitory effect of propranolol on response to isoprenaline in lymphocytes from 2 normal subjects (□ and ●) and one with chronic lymphatic leukaemia (△). Values for $pA_2$ and slope of plot respectively are as follows: (□) 8.38; 2.2; (O) 8.34; 2.0; (△) 8.32; 1.8. (b) Schild plot showing the inhibitory effect of practolol on response to isoprenaline in lymphocytes from 2 normal subjects. Values for $pA_2$ and slope of plot respectively are as follows:- (▲) 4.0; 3.2; (●) 3.89; 2.6.

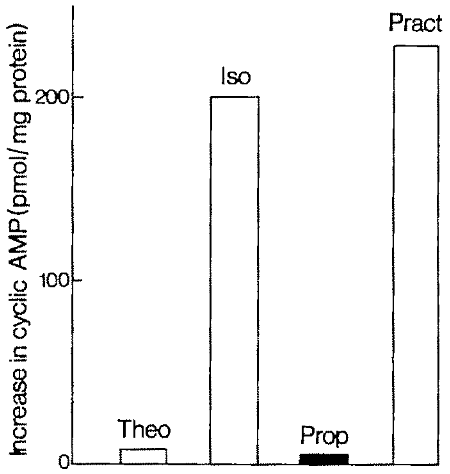

**Figure 7** Cyclic AMP response (pmol/mg protein) to theophylline (Theo), isoprenaline plus theophylline (Iso), isoprenaline and theophylline plus propranolol (Prop) and isoprenaline and theophylline plus practolol (Pract). Concentrations used were: theophylline $10^{-2}$ mol/l; isoprenaline $10^{-4}$ mol/l; propranolol $10^{-4}$ mol/l and practolol $10^{-4}$ mol/l. Incubations were for 15 min at 37°C.

values ranged from 8.32 to 8.38 for propranolol, and 3.89 to 4.00 for practolol. Values obtained in other tissues in several laboratories are given in Table 1 for comparison.

β-Receptors in lung parenchyma appear to respond in the same way as those of lymphocytes with respect to propranolol and practolol. Figure 7 shows the response of duplicate samples of lung tissue to isoprenaline ($10^{-4}$ mol/l), isoprenaline plus propranolol ($10^{-4}$ mol/l of each drug) and isoprenaline plus practolol ($10^{-4}$ mol/l of each drug). Propranolol virtually abolished the effect of isoprenaline, whereas with practolol there is a slight further increase, from 196.6 to 232.0 pmol cyclic AMP per mg protein. No attempt has been made to determine $pA_2$ values for lung parenchyma because of the heterogeneous cell population involved.

**Table 1** $pA_2$ values for propranolol and practolol obtained in other laboratories

| *Tissue* | *Propranolol* | *Practolol* | *Reference* |
|---|---|---|---|
| Guinea-pig atria | 8.70 | | Blinks, 1967 |
| | 8.02 | | Moore & O'Donnell, 1970 |
| | 7.98 | | Bassett, 1971 |
| | 8.71 | | Conolly, 1972 |
| | 8.32 | 6.49 | Barrett *et al.*, 1973 |
| Guinea-pig trachea | 8.02 | | Moore & O'Donnell, 1970 |
| | 7.93 | | Bassett, 1971 |
| | 8.46 | 4.26 | Barrett, Carter, Fitzgerald, Hull & Le Count, 1973 |
| Human bronchus | 6.65 | 4.29 | Hedges & Turner, 1971 |

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## Discussion

The subdivision of adrenoceptors into two broad categories, $\alpha$ and $\beta$, first suggested by Ahlquist (1948), is now well established. There is much less agreement on the validity of the further subdivision of $\beta$-adrenoceptors into $\beta_1$ and $\beta_2$ subgroups (Lands *et al.*, 1967) principally because there seem to be too many receptors which will not fit neatly into either group (Bristow, Sherrod & Green, 1970; Lefkowitz, 1975).

Nevertheless, it remains a clinically useful concept since it has been possible to develop relatively cardioselective ($\beta_1$) adrenoceptor antagonists such as practolol, atenolol (ICI 66082, Tenormin), and metoprolol, which may be used to treat angina or hypertension in many, though not all, patients with obstructive airways disease. Conversely selective $\beta_2$-agonists such as salbutamol and terbutaline are being used increasingly in the treatment of bronchial asthma, since they produce the same bronchodilatation as isoprenaline but with much less tachycardia.

It is desirable to measure the results of receptor stimulation as directly as possible, and in this respect the identification of cyclic AMP as an intracellular 'second messenger' has been of great value. However, for cyclic AMP to provide a reliable index of receptor stimulation from one experiment to another, a homogeneous cell population must be studied, and in an attempt to achieve this, we decided to use lymphocytes, which have higher levels of adenylate cyclase activity than granulocytes. However, lymphocyte separation on a density gradient, used here because of its advantages of speed and simplicity, may result in a selective loss of T lymphocytes (Thompson & Vaughan Smith, personal communication). Bach (1975) has shown in the mouse that T and B lymphocytes differ markedly from one another in respect of adenylate cyclase activity and this may well explain the pattern of variability described above. Since cells with low basal activity respond least well to $\beta$ stimulants and *vice versa,* it was felt preferable to analyse the data as proportional (percentage) increase in cyclic AMP.

We have shown that the lymphocyte $\beta$-adrenoceptor is readily blocked by (±)-propranolol, but appears to be far less sensitive to the effect of practolol. The same pattern of susceptibility to the different $\beta$-blockers is seen in lung tissue.

This difference in potency between practolol and propranolol is, like that reported by Dunlop & Shanks (1968), greater than the difference observed by Vaughan Williams, Bagwell & Singh (1973) in anaesthetized dogs. The latter group reported a potency ratio of 75.9:1 in favour of propranolol when examining blockade of isoprenaline-induced vasodilatation. The difference between the results of Vaughan Williams *et al.* and those reported here may in part be due to species difference. However, it is possible that differences in the experimental conditions used also played a part. In the present study, the cells were suspended in an aqueous medium (a relatively polar solvent system), whereas in the dog studies the tissues were bathed with blood, which has a high lipid content. Practolol is far more polar than propranolol, and in experiments using aqueous media, it will preferentially partition into the medium rather than into the lipid of the cell membrane, and this may hinder access to the receptors. For the lipid soluble propranolol the reverse will be the case, tending thereby to exaggerate the already substantial difference in the potency of the two drugs with respect to the $\beta_2$-receptor. The $pA_2$ values for both propranolol and practolol correspond closely to those obtained by most other authors and conform to the pattern which would be predicted for a tissue possessing a $\beta_2$receptor. The slopes of the Schild plots are greater than unity (the expected value for a true competitive antagonism). There is no obvious explanation for this. Both propranolol and practolol have been shown in other tissues to exert only a simple competitive antagonism, and studies with another $\beta$-blocking drug, alprenolol, in human lymphocytes (Williams, Snyderman & Lefkowitz, 1976) indicate that $\beta$-blockade is a reversible competitive antagonism in this cell model.

The response to salbutamol is in keeping with the pattern of response to the antagonist drugs. Salbutamol stimulated the lymphocyte $\beta$-adrenoceptor, being about one tenth as active as isoprenaline. These findings correspond closely to those of Cullum, Farmer, Jack & Levy (1969) who reported a 10:1 potency ratio between isoprenaline and salbutamol on guinea-pig tracheal chain. These authors found in contrast a 2000:1 potency ratio with respect to cardiac $\beta$-receptors in the same species. In the lymphocyte, unlike tracheal smooth muscle, salbutamol acts as a partial agonist.

On the basis of these studies the lymphocyte adrenoceptor may (within the limitations of this classification) be categorized as $\beta_2$ and in this respect is valid as a model for studying $\beta$-receptor function in asthma.

Assessment of $\beta_2$-adrenoceptor function by non-invasive methods is not easy. However, the lymphocyte offers a very accessible receptor system and as such may be a useful tool for measuring this, and for assessing the selectivity of $\beta$-adrenoceptor antagonists (and possibly also selective drugs of other types) in man.

This work was supported by a grant from the Medical Research Council. The authors acknowledge gratefully the advice, encouragement and helpful criticism of Professor C.T. Dollery during the conduct of these studies. They are grateful to Mr P. Schofield for skilled technical assistance. Practolol and propranolol were generously dontated by ICI Ltd. and salbutamol sulphate by Allen and Hanbury's Ltd.

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*(Received February 2, 1976.*
*Revised June 16, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 25–28

# DOPAMINE AND THE DEPRESSANT ACTION OF MORPHINE ON STIMULATED GUINEA-PIG ILEUM

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1 Morphine reduces the amplitude of the contractions induced by electrical stimulation, in the myenteric plexus-longitudinal muscle preparation of guinea-pig ileum. Dopamine and apomorphine have the same effect but at much higher concentrations.

2 Dopamine, at concentrations lower than those which would normally be inhibitory, partially reverses the depressant effect of morphine.

3 Pre-treatment of guinea-pigs with 6-hydroxydopamine results in a slight supersensitivity of innervated longitudinal muscle preparations to dopamine and has no effect on morphine activity.

4 Naloxone antagonizes the depressant effect of morphine but not that of dopamine or apomorphine.

5 The response of the ileum preparation to morphine is not affected by phentolamine or propranolol; the effect of dopamine, however, is abolished by $\alpha$-adrenoceptor blockade.

## Introduction

Morphine decreases the amplitude of the electrically-evoked contractions in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum (Paton, 1957; Gyang & Kosterlitz, 1966). This test has been used in order to study the basic mechanism of the effects of narcotic analgesics; since it has been shown that the receptors of the myenteric plexus are like those in the central nervous system (Fennessey, Heimans & Rand, 1969; Ehrenpreis, Light & Schonbuch, 1972). A reduced release of acetylcholine is considered responsible for the effect of morphine on the ileum (Cox & Weinstock, 1966) while an efflux of noradrenaline is generally excluded (Gyang & Kosterlitz, 1966; Kosterlitz & Watt, 1968) since $\alpha$- and $\beta$-adrenoceptor blocking drugs do not modify the response of the ileum to narcotic agonists. It is also possible that morphine, in addition to the prevention of the release of acetylcholine (an excitatory neuro-transmitter substance in the gut), may cause a release of an inhibitory modulator of transmission; this substance may be dopamine as suggested by Goldstein & Schulz (1973). On the other hand, there is evidence to support the possibility of a relationship between dopamine and some effects of morphine in the central nervous system (Fennessy & Lee, 1972). We have, therefore, carried out this investigation in which the effects of dopamine and of the dopaminergic drug, apomorphine were examined and compared with the effect of morphine on the myenteric plexus-longitudinal muscle preparation of ileum taken from normal guinea-pigs and guinea-pigs pre-treated with 6-hydroxydopamine; the latter drug causes selective destruction of catecholamine-containing nerve terminals (Sachs & Jonsson, 1975).

## Methods

### *Animal pretreatment*

Guinea-pigs of either sex weighing between 300 and 500 g were given an intraperitoneal injection of 30 mg/kg 6-hydroxydopamine dissolved in 0.9% w/v NaCl solution (saline) containing ascorbic acid (1 mg/ml) 36 h before they were killed.

Control animals received only the solvent.

### *Ileum preparation*

The myenteric plexus-longitudinal muscle preparation from the guinea-pig (Paton & Vizi, 1969) was set up in a 5 ml organ bath containing Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $KH_2PO_4$ 1.19, $NaHCO_3$ 25, glucose 11, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and choline chloride 20 µM. A platinum ring electrode (anode) was placed at the top of the bath; the cathode was a platinum hook at the bottom of the bath. Electrical rectangular pulses were used of 1 ms duration, 0.2 Hz frequency and supramaximal (80 V) voltage (1.3 times the maximal voltage). Before addition of drugs to the bath fluid, the preparations were allowed to equilibrate for at least 60 min, with renewal of the bath fluid every 10 minutes.

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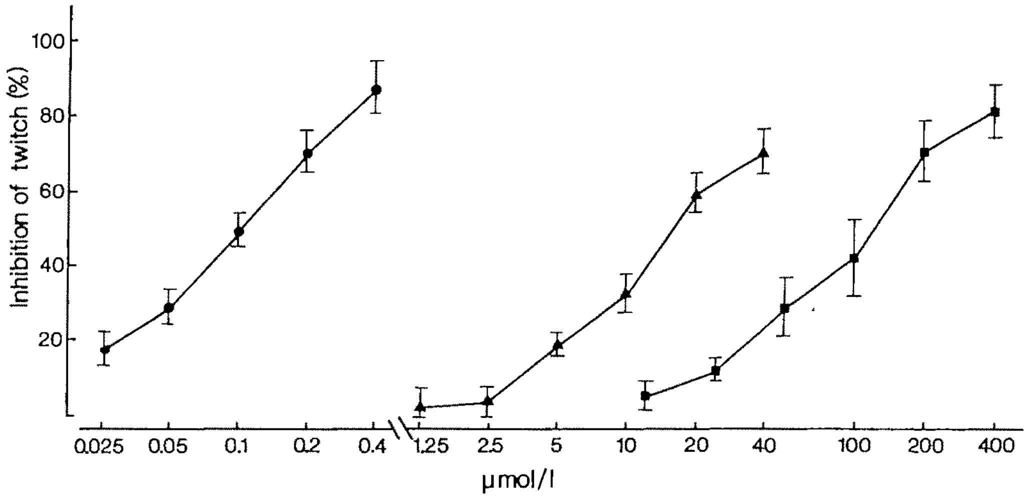

**Figure 1** Inhibitory effects of morphine (●), dopamine (▲) and apomorphine (■) on the electrically induced twitches of the longitudinal muscles of guinea-pig ileum: each point represents the mean of 6 experiments. Vertical lines show s.e. mean.

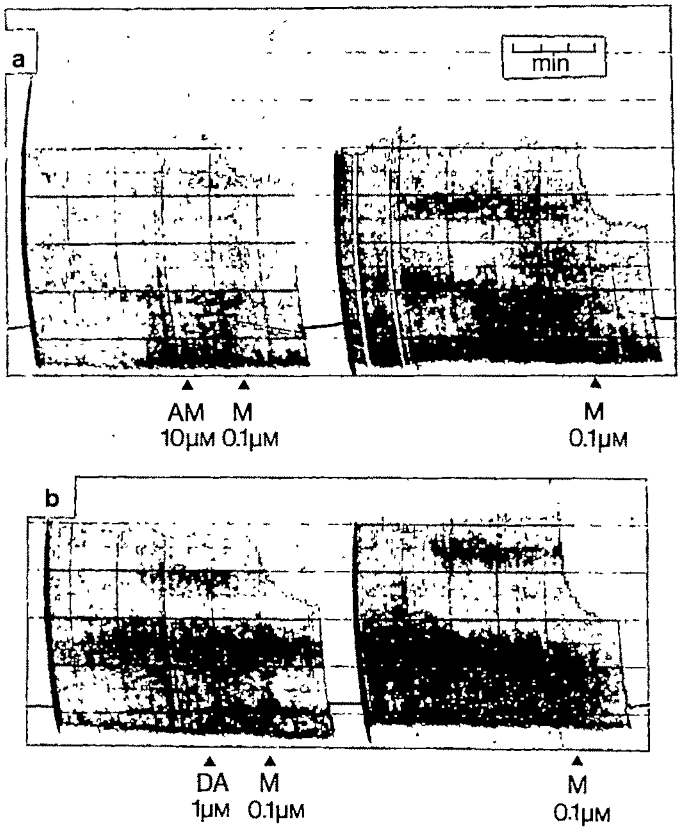

**Figure 2** Antagonism by low concentrations of (a) apomorphine (AM) and (b) dopamine (DA) of the inhibitory effects of 100 nM morphine (M) on the electrically induced twitches of guinea-pig myenteric plexus-longitudinal muscle preparation.

### *Drugs*

The drugs used were: morphine hydrochloride (C. Erba), dopamine hydrochloride (Sigma), apomorphine hydrochloride (Biosintex), naloxone hydrochloride (Endo), phentolamine hydrochloride (Ciba), propranolol hydrochloride (ICI), 6-hydroxydopamine hydrobromide (Hoffman La Roche). All drug concentrations are expressed in terms of the base.

### *Statistical analysis*

Student's $t$-test was calculated in the usual manner (Steel & Torrie, 1960).

## Results

### *Effect of morphine, dopamine and apomorphine on the responses of the longitudinal muscle to electrical stimulation*

The amplitude of contraction (twitches) of electrically stimulated myenteric plexus-longitudinal muscle preparations was reduced by all three drugs (Figure 1); 50% depression of twitches required final bath concentrations of 100 nM morphine, 16 μM dopamine and 120 μM apomorphine. When smaller concentrations of dopamine (1 μM) or apomorphine (10 μM) were added to the bath fluid, approximately 2 min before morphine (100 nM), the inhibitory effect of the analgesic was reduced (Figure 2) but was never completely abolished; higher concentrations of dopamine and apomorphine could not be used owing to their depressant effect on the ileum.

### *Effect of 6-hydroxydopamine on the response of the ileum to morphine and dopamine*

The depressant action of morphine on the electrically stimulated ileum was not affected by pretreatment of guinea-pigs with 6-hydroxydopamine; however, the preparation showed an increased sensitivity to dopamine. In a separate series of six experiments, it was found that the maximal (percentage) inhibition of the twitch produced to 10 μM dopamine was significantly ($P<0.05$) increased from 33.97±4.50 (mean with s.e. mean) in controls to 52.61±5.20 (mean with s.e. mean) in ilea from guinea-pigs pretreated with 6-hydroxydopamine.

### *Effects of antagonists*

Naloxone abolished the depressant effect of morphine (100 nM) at a concentration of 10 nM but not that of dopamine (10 μM) or apomorphine (100 μM) even at higher concentrations (Table 1). The $\alpha$-adrenoceptor antagonist, phentolamine, in concentrations of 355 nM and 1 μM, abolished the effect of dopamine but not that of morphine. The $\beta$-adrenoceptor antagonist, propranolol, did not alter the response to any of the tested drugs (Table 1).

**Table 1** Effect of naloxone and of adrenoceptor blocking drugs on the action of morphine (100 nM), dopamine (10 μM) and apomorphine (100 μM) on the responses of myenteric plexus-longitudinal muscle preparation to electrical stimulation

| *Inhibiting drug* | *Antagonist* | *No. of expts* | *Inhibition of twitch (%)* *Without antagonist* | *With antagonist* | P[1] |
|---|---|---|---|---|---|
| Morphine | Naloxone 10 nM | 5 | 47.92±4.22 | 0.46±1.01 | <0.01 |
| Morphine | Phentolamine 355 nM | 6 | 49.63±4.20 | 48.89±3.94 | >0.05 |
| Morphine | Phentolamine 1 μM | 6 | 48.45±5.10 | 41.88±3.65 | >0.05 |
| Morphine | Propranolol 1.2 μM | 6 | 49.93±5.63 | 52.10±6.15 | >0.05 |
| Morphine | Propranolol 4 μM | 6 | 51.11±7.38 | 45.29±7.18 | >0.05 |
| Dopamine | Naloxone 10 nM | 6 | 40.79±3.41 | 41.31±1.44 | >0.05 |
| Dopamine | Naloxone 100 nM | 6 | 38.42±5.21 | 37.41±6.01 | >0.05 |
| Dopamine | Naloxone 1 μM | 6 | 42.05±6.60 | 41.90±6.12 | >0.05 |
| Dopamine | Phentolamine 355 nM | 6 | 37.20±7.71 | 5.66±1.69 | <0.01 |
| Dopamine | Phentolamine 1 μM | 6 | 35.58±5.72 | 0.88±2.40 | <0.01 |
| Dopamine | Propranolol 1.2 μM | 6 | 35.78±6.19 | 34.95±8.73 | >0.05 |
| Dopamine | Propranolol 4 μM | 6 | 31.14±5.15 | 29.29±4.37 | >0.05 |
| Apomorphine | Naloxone 10 nM | 6 | 32.43±5.62 | 31.14±4.23 | >0.05 |
| Apomorphine | Naloxone 1 μM | 6 | 28.17±5.43 | 30.24±4.48 | >0.05 |

[1] Compared with experiments without antagonist by $t$-test.

## Discussion

The potency of morphine in reducing the amplitude of contractions induced by electrical stimulation in the longitudinal muscle preparation of ileum taken from guinea-pigs was not affected by pretreatment with 6-hydroxydopamine. We have confirmed the inhibitory effect of dopamine at high concentrations (Goldstein & Schulz, 1973), and shown that it is present, and even increased, in 6-hydroxydopamine-treated preparations.

These findings suggest that the action of morphine is not mediated by a release of endogenous dopamine in the intestine. In contrast, Heimans (1975) found a reduced activity of morphine in this preparation from guinea-pigs pretreated with reserpine. However, it is possible that the effect of reserpine is less specific. It is more likely that morphine and dopamine, mimicking morphine, act on separate receptors that are independent of each other. In fact, only a specific inhibitor of analgesics, naloxone, abolished the action of morphine on twitches while the blockade of the adrenoceptors was wholly ineffective; contrariwise, the inhibitory action of dopamine seems mediated by $\alpha$-adrenoceptor stimulation since it was inhibited by phentolamine. These findings are in agreement with those of Kosterlitz & Watt (1968) who showed that the depressant action of morphine on the ileum is unimpaired after blockade of $\alpha$- and $\beta$-adrenoceptors, and also with the data of Heimans (1975) who showed that haloperidol, an antagonist of dopamine receptors also in peripheral tissues (Yeh, McNay & Goldberg, 1969), does not alter the action of the narcotic agonist on the ileum. On the other hand, dopamine and apomorphine can mimic the effect of morphine on guinea-pig ileum preparations only at concentrations which are much higher than those of morphine.

The apparent antagonism of morphine by dopamine and apomorphine in low concentrations is not readily explained. However, in this context, some recent unpublished observations on analgesia in rats are of interest; depending on the dose, dopamine administered intra-cerebroventricularly had both morphine-like and morphine-inhibitory effects.

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*(Received February 20, 1976.*
*Revised June 25, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 29–33

# THE EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON ADENOSINE TRIPHOSPHATE CONTENT AND HISTAMINE RELEASE FROM RAT PERITONEAL CELL SUSPENSIONS RICH IN MAST CELLS

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1 Non-steroidal anti-inflammatory drugs (NSAID) suppressed compound 48/80-induced histamine release from rat peritoneal cells *in vitro* in a dose-dependent manner.

2 NSAID suppressed the adenosine triphosphate (ATP) content of rat peritoneal cells *in vitro* and this correlated strongly with the suppression of compound 48/80-induced histamine release.

3 The correlation demonstrated suggests that the mechanism of action of NSAID in the rat peritoneal cells is via depletion of cellular ATP.

## Introduction

Mast cells, which are rich in histamine and other vasoactive factors, are involved in the immediate hypersensitivity reaction (Mongar & Schild, 1962; Austen, 1974) and probably in the early stages of acute inflammation (Keller, 1968). The release of these factors can be initiated by antigen directed against specific cell bound antibodies, some products of activated complement (Dias da Silva & Lepow, 1968; Cochrane & Muller-Eberhard, 1968) ATP (Diamant & Kruger, 1967) compound 48/80, various basic drugs (Goth, 1967) and dextrans (Goth, 1966; Baxter, 1972). These agents can release without concomitant cell disruption or loss of intracellular constituents, such as potassium ions or lactic dehydrogenase (Johnson & Moran, 1969). The binding of endogenous or exogenous releasing agents to specific surface receptors on the mast cell membrane (Bach & Brashler, 1973; Read, Ortner, Hino & Lenney, 1974) initiates a sequence of ill-defined intracellular events culminating in granule exocytosis. This exocytosis requires energy (Diamant & Uvnäs, 1961; Uvnäs, 1963) and generally calcium (Foreman & Mongar, 1972); it may be modulated by intracellular levels of cyclic 3′,5′-AMP (Sullivan, Parker, Eisen & Parker, 1975). In a second phase histamine and other factors are displaced from the released granules by cations from the extracellular fluid (Thon & Uvnäs, 1967).

Non-steroidal anti-inflammatory drugs (NSAID) such as indomethacin, aspirin and phenylbutazone, suppress the release of histamine from rat mast cells by compound 48/80 and antigen (Yamasaki & Saeki, 1967; Norn, 1971). This paper describes the relationship between histamine release and ATP content in rat peritoneal cells incubated with several NSAID.

## Methods

Male Fullensdorf Albino rats weighing 200–300 g were used in all experiments. Mixed peritoneal cells were collected by the method of Johnson & Moran (1966). Mast cells comprised 7.2 ± 0.8% (mean ± s.e. mean) of the mixed peritoneal cell population. They were not separated from other cells as the histamine from rat peritoneal cells is contained in the mast cells (Norn, 1968; Herzig & Kusner, 1975). Pooled cells from six rats were resuspended in the buffered salt solution to give 100,000–200,000 mast cells/ml. All experiments were performed both with and without dextrose (5.6 mM) in the media. Aliquots of the cell suspension (0.5 ml) were pre-incubated at 37°C with the drug to be tested which was added in 0.25 ml of the buffered salt solution at pH 7.0. Aliquots (50 μl) of the incubation suspension were added to 1.95 ml of TRIS (hydroxymethyl) -methyl-amine buffer (0.02 M, pH 7.75) at 100°C for 15 s, then plunged into an ice bath and later stored at −20°C until assayed for ATP. Various concentrations of compound 48/80 (Burroughs Wellcome & Co.) dissolved in 0.25 ml of the buffered salt solution were incubated with the cell suspension for 5 minutes. The suspension was then centrifuged at 300 *g* for 5 min at 4°C. Histamine in the supernatant was assayed by the method of Shore, Burkhalter & Cohn (1959) as modified by Kremzner & Wilson (1961) omitting the butanol extraction procedure for histamine which is unnecessary in this system (Loeffler, Lovenberg & Sjoerdsma, 1971). The total histamine content was measured by disrupting the cells (0.5 ml cell suspension diluted to 0.95 ml) by boiling for 10 min and then assaying the histamine in the supernatant. Histamine release was expressed as a percentage of the histamine released on cell disruption. Oxyphenbutazone was the only drug examined that affected the histamine assay and histamine standards were assayed in the presence of appropriate concentrations of oxyphenbutazone.

ATP levels were measured by a modification of the method of Cheer, Gentile & Hegre (1974). Buffered firefly lantern extract (Sigma Chemical Company) reconstituted with double distilled water was placed in an ice bath 30–60 min before use. The samples for ATP assay were thawed and also kept at 0°C. Using uniformly chilled glass scintillation vials containing a glass reaction cuvette, 200 µl of the unknown samples were quickly added to 150 µl of firefly tail extract suspension which had been standing in the cuvette for 60 s exactly. After 30 s, the light emitted was measured for 60 s in a 'tray loaded' Phillip's Liquid Scintillation Analyser. (The accurate timing described was necessary to standardize the temperature of the luciferin-luciferinase reaction and because of the mechanics of the counter.) ATP dissolved in the TRIS buffer and boiled for 15 s was used for standards. To correct for drug effects on this assay, ATP standards were measured in the presence of the drugs at each concentration studied. Log-log plots gave linear standard curves.

The percentage reduction of ATP content (A. Sup.) of the mixed peritoneal cells induced by the various concentrations of drugs and the percentage suppression of histamine release (H. Sup.) were calculated from the equations:

$$\text{H. Sup.} = \frac{A_H - B_H}{A_H} \times 100$$

and,

$$\text{A. Sup.} = \frac{A_{ATP} - B_{ATP}}{A_{ATP}} \times 100$$

where: $A_H$ = maximum percentage release of histamine by compound 48/80, $B_H$ = maximum percentage release of histamine by the same concentration of compound 48/80 from cells preincubated with a NSAID, $A_{ATP}$ = concentration of ATP in ng per ml of the peritoneal cell suspension incubated at 37°C and $B_{ATP}$ = concentration of ATP in ng per ml of the peritoneal cell suspension preincubated with a NSAID at 37°C.

The NSAID studied were all obtained as pure substances. Indomethacin was donated by Merck, Sharpe & Dohme (Australia), Ro 20–5720 by Roche Products (Australia), ibuprofen and fluorbiprofen by the Boots Company (Australia), and naproxen by Syntex Corporation.

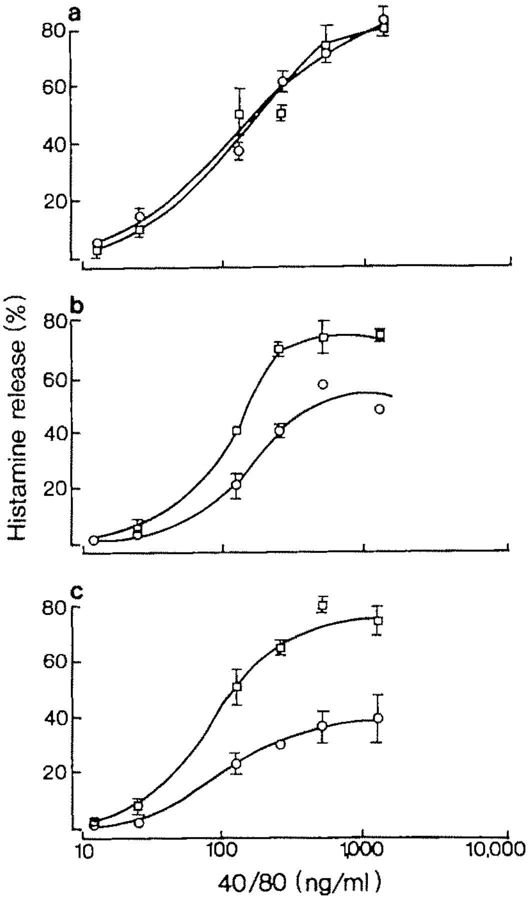

**Figure 1** The effect of increasing concentrations of indomethacin on dose-response curves of histamine release induced by compound 48/80. In this representative experiment, dextrose was absent from the media. The cells were incubated with (a) indomethacin (○) 0.85 mM (b) 1.0 mM and (c) 1.4 mM or without indomethacin (□). Compound 48/80 was added after 30 min incubation. Each point is the mean of three histamine estimations. Vertical lines show s.e. means. There were significant differences ($P < 0.05$, unpaired Student's $t$ test) between the test cells in experiments (b) and (c) and their respective control cells for all concentrations of 48/80 greater than 100 ng/ml.

## Results

### *Histamine release and ATP content of rat peritoneal cells incubated with NSAID*

NSAID suppressed the 48/80-induced release of histamine from rat peritoneal cells in a dose-dependent fashion (Figure 1). The percentage suppression of histamine release and the corresponding percentage reduction in ATP content at each concentration of NSAID (Table 1) showed a high degree of correlation ($r=0.82$, $P<0.001$) when all experiments were considered together. This correlation remained highly significant when incubations with or without dextrose were examined separately (dextrose 5.6 mM, $r=0.74$, $P<0.001$: no dextrose, $r=0.85$, $P<0.001$).

The absence of dextrose from the media generally

**Table 1** The mean percentage suppression of histamine release (±s.e. mean) induced by compound 48/80 (300 ng/ml) and the mean percentage reduction of ATP content (±s.e. mean) of rat peritoneal cells preincubated with various non-steroidal anti-inflammatory drugs for 30 minutes

| | *Dextrose (5.6 mM)* | | *No dextrose* | |
|---|---|---|---|---|
| *Drug (mM)* | *% Histamine suppression* | *% ATP reduction* | *% Histamine suppression* | *% ATP reduction* |
| Flufenamic acid | | | | |
| 0.05 | | | 52.0±3.7 | 72.1±2.1 |
| 0.07 | 32.0±0.1 | 41±11.8 | | |
| 0.09 | | | 100±0.03 | 82±6.2 |
| 0.10 | 100±0.05 | 84±1.0 | | |
| Ibuprofen | | | | |
| 1.0 | | | 24±2.3 | 6±9.0 |
| 1.5 | | | 96±2.3 | 74.7±2.1 |
| 2.2 | 35±4.3 | 29.6±23.8 | 100±1.3 | 100±0.2 |
| 2.4 | 89±3.1 | 51±3.0 | | |
| Indomethacin | | | | |
| 0.7 | 18±4.2 | 33.1±5.6 | −7.0±11.4 | 20.1±5.3 |
| 1.0 | 32±0.8 | 61.5±9.7 | | |
| 1.4 | | | 32±4.9 | 38.4±8.5 |
| RO 20–5720 | | | | |
| 0.09 | | | 3.0±8.5 | 14.8±8 |
| 0.18 | 35.1±0.1 | 32±5.8 | 69.6±2.0 | 62±1.1 |
| 0.36 | 72.6±0.1 | 48.5±11.3 | 75.8±1.0 | 76±7.0 |
| Oxyphenbutazone | | | | |
| 0.15 | 6.7±0.1 | −12±12.3 | 1.1±0.1 | −18±1.1 |
| 0.30 | 51.3±5.0 | 48.1±4.5 | 46.2±0.1 | 12±5.2 |
| Phenylbutazone | | | | |
| 0.4 | | | −0.1±0.3 | 53.8±3.9 |
| 0.7 | | | 98.0±1.2 | 74±1.9 |
| 1.3 | 6.8±0.05 | 45±4.4 | | |
| 1.6 | 58.0±0.4 | 49±9.8 | | |
| Fluorbiprofen | | | | |
| 0.6 | | | 8.8±5.9 | 20.4±6.7 |
| 1.0 | 46.7±2.3 | 56.8±10.6 | 100±0.2 | 83.7±7.9 |
| Acetylsalicylic acid | | | | |
| 2.8 | | | −0.7±4.6 | −18±9.2 |
| 3.3 | 1.9±0.5 | 2.4±6.5 | 61.3±1.0 | 49±2.5 |
| 5.0 | 19.4±0.1 | 8.8±2.7 | | |
| 5.7 | 23.1±0.7 | −1.0±8.2 | | |
| Salicylic acid | | | | |
| 7.3 | | | 36.2±0.7 | 40±4.7 |
| 10.8 | 11.7±0.5 | 23±5.8 | | |
| Naproxen | | | | |
| 2.2 | 2.5±0.8 | 0.05±0.06 | 20.2±0.5 | 12.6±0.04 |
| 3.3 | 19.8±3.6 | 17.5±6.0 | 41.7±0.5 | 23.6±4.7 |
| 4.4 | | | 25.8±0.04 | 24.0±4.0 |
| 5.4 | 50.6±3.8 | 18.0±4.4 | | |

made the mast cells more sensitive to the inhibitory effects of NSAID, particularly, of phenylbutazone, flufenamic acid, ibuprofen, acetylsalicylic acid and salicylic acids (Table 1).

## Discussion

This study and those of Norn (1971) and Yamasaki & Saeki (1967) indicate that NSAID suppress 48/80 and antigen-induced histamine release from rat mast cells. The rank order of potency of NSAID in this system is similar to that found using various *in vitro* and *in vivo* tests for anti-inflammatory activity (Wax, Winder, Tessman & Stephens, 1974). One discrepancy was the finding that oxyphenbutazone was more potent than phenylbutazone in this cell system, although these drugs have similar anti-inflammatory potencies *in vivo*. This phenomenon has been noted in other test systems (Takagi & Fukao, 1971) but is unusual (Ignarro, 1971; Clark, 1972).

The concentrations of some of the NSAID required to inhibit histamine release from the peritoneal cells are higher than the presumed effective concentrations *in vivo*. However, these drugs accumulate at sites of inflammation (Graf, Glatt & Brune, 1975). Also the sensitivity of tissue mast cells to NSAID may be greater *in vivo* than *in vitro*. It appears that the rat peritoneal cell system can give useful information on new anti-inflammatory compounds and on the mode of action of NSAID.

The observed correlation between the suppression of histamine release and the reduction in peritoneal cell ATP suggests that NSAID may act in this system by lowering cellular ATP levels. The liberation of histamine by antigen or compound 48/80 from rat mast cells and other tissues such as guinea-pig lung is an energy-dependent process which can be blocked by anoxia and metabolic inhibitors such as 2,4-dinitrophenol, oligomycin and cyanide (Parrat, 1942; Mongar & Shild, 1957). This blocking effect is antagonized by glucose. Using metabolic inhibitors to manipulate cellular ATP levels, Johansen & Chakravarty (1972; 1975) have shown that the amount of histamine released from rat mast cells by compound 48/80 or by the anaphylactic reactions, bears a linear relationship to the ATP content of the cells prior to challenge with either of these histamine releasers. Peterson & Diamant (1973) demonstrated that exposure of rat mast cells to compound 48/80 consumed 25–30% of their ATP content. There is no conclusive evidence yet that ATP is utilized in the histamine release from sensitized mast cells initiated by antigen (Diamant, 1975).

The NSAID, although chemically heterogeneous, have the common biochemical action of uncoupling oxidative phosphorylation (Whitehouse, 1968). This effect is possibly a consequence of drug binding to essential amino groups in the mitochondrial energy chain (Whitehouse, 1964; 1968) and its potency *in vitro* parallels the anti-inflammatory potency of NSAID *in vivo*. Additionally, the inhibitory actions of NSAID in the rat peritoneal cell system were enhanced when dextrose, a major source of cellular energy, was absent. Therefore, it seems likely that NSAID reduced the ATP content of the cells by uncoupling oxidative phosphorylation. The ATP content probably determined the amount of histamine released by compound 48/80. ATP levels may similarly influence the release by antigen.

It should be noted that the changes in ATP levels measured in this study relate to the total peritoneal cell population and possibly may not reflect the ATP levels of the mast cell subpopulation. However, it seems unlikely that the NSAID would differentiate between the mast cells and the other peritoneal cells since the NSAID uncouple oxidative phosphorylation in many *in vitro* systems and are well known to interact with a wide variety of cell and organelle membranes (Famey & Whitehouse, 1975).

The role of the uncoupling of oxidative phosphorylation (Kalben & Domenjoz, 1968) or inhibition of histamine release in the actions of NSAID *in vivo* is not yet clear. However, since all NSAID tested produced these effects the rat peritoneal cell model may be a useful method for the study of compounds with anti-inflammatory activity.

We would like to thank Miss Louise Dennis for Secretarial help. R.O.D. is supported by the National Health and Medical Research Council of Australia. G.D.C. is Honorary Physician to St. Vincent's Hospital, Darlinghurst, N.S.W., Australia.

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*(Received February 24, 1976.*
*Revised May 24, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 35–41

# STRUCTURE-ACTIVITY RELATIONSHIP OF VARIOUS CORTICOSTEROIDS ON THE FEEDBACK CONTROL OF CORTICOTROPHIN SECRETION

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**1** Several steroids occurring in the pathway of corticosteroid biosynthesis were investigated for their ability to exert a fast or delayed feedback inhibition of stress-induced release of corticotrophin. Rats were injected subcutaneously with vehicle or a steroid either 10 min (fast feedback) or 4 h (delayed feedback) before they were subjected to stress which consisted of a 2 min exposure to ether vapour.

**2** Changes in plasma corticosterone concentration and *in vitro* corticosterone production by excised adrenal glands were used as indices of corticotrophin release.

**3** Among the steroids tested only 11$\beta$, 21-dihydroxypregn-4-ene-3, 20-dione (corticosterone) and 11$\beta$, 17$\alpha$, 21-trihydroxypregn-4-ene-3, 20-dione (cortisol) inhibited the stress response 10 min after their administration. Therefore, it appears that the fast feedback mechanism is limited to steroids with a 21-hydroxyl and a 11$\beta$-hydroxyl group.

**4** In contrast, many steroids caused inhibition of the stress response 4 h after their administration. These steroids were corticosterone, cortisol, 21-hydroxypregn-4-ene-3, 20-dione (11-deoxycorticosterone), 17$\alpha$, 21-dihydroxypregn-4-ene-3, 20-dione (11-deoxycortisol), 11$\beta$-hydroxypregn-4-ene-3, 20-dione (11$\beta$-hydroxyprogesterone) and 11$\beta$, 17$\alpha$-dihydroxypregn-4-ene-3, 20-dione (11$\beta$, 17$\alpha$-dihydroxyprogesterone). Thus, either the 21-hydroxyl group (e.g. 11-deoxycorticosterone) or the 11$\beta$-hydroxyl group (e.g. 11$\beta$-hydroxyprogesterone) is sufficient for delayed feedback activity. The 11$\alpha$-hydroxyl group, e.g. 11$\alpha$, 17$\alpha$, 21-trihydroxypregn-4-ene-3, 20-dione (11-epicortisol) renders the steroid inactive on both feedback mechanisms.

**5** 18, 21-Dihydroxypregn-4-ene-3, 20-dione (18-hydroxydeoxycorticosterone) was found to be the only steroid that is secreted by the adrenal gland of the rat in quantities sufficient to cause exaggeration of the stress-induced release of corticotrophin. This steroid has been implicated as a possible hypertensive agent, and its role in the control of corticotrophin secretion is discussed here.

## Introduction

There are two temporally distinct periods of inhibition of stress-induced release of corticotrophin (ACTH) following pretreatment with corticosteroids; these have been designated fast and delayed feedback (Dallman & Yates, 1969). Fast corticosteroid feedback occurs immediately after steroid administration and coincides with the time when the plasma steroid concentrations are rising (Dallman & Yates, 1969; Jones, Brush & Neame, 1972). Delayed corticosteroid feedback occurs an hour or more after steroid administration when the plasma steroid concentrations may have returned to basal values (Smelik, 1963; Hodges & Sadow, 1967; Dallman & Yates, 1969).

The steroids 11-deoxycorticosterone and 11-deoxycortisol have been found to have different actions on the fast and delayed feedback mechanisms; in fairly high doses they interfere with the fast feedback mechanism of corticosterone, but they cause inhibition of corticotrophin release when injected some hours before exposure to stress (Jones, Tiptaft, Brush, Fergusson & Neame, 1974). In view of the proposed separate receptors involved in these two feedback mechanisms, we have tested several steroids from the corticosteroid biosynthetic pathway for 'fast or delayed feedback actions. Some of the findings have been briefly reported previously (Tiptaft & Jones, 1975).

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## Methods

Male Wistar-derived rats, weighing 140–200 g from a pathogen-free colony were kept three per cage in a sound insulated room for at least 12 h before each experiment. Lights were on from 07 h 00 min to 21 h 00 min and all experiments were performed between 08 h 00 min and 14 h 00 min, when the plasma corticosterone concentration was low and relatively constant.

### *Stress*

The stress employed was a 2 min exposure to ether vapour; 25–30 min afterwards the animals were decapitated and blood was collected for the estimation of plasma corticosterone. At the same time one adrenal gland was removed, weighed, quartered and then incubated in 2 ml Krebs-Ringer solution at 37°C in an atmosphere of 95% $O_2$ and 5% $CO_2$ for one hour. The corticosterone content of the incubation medium was then measured.

### *Injections*

In the experiments in which the fast feedback mechanism was tested, the animals were injected 10 min before ether inhalation with a 0.9% sodium chloride solution (saline s.c.), or a suspension of a steroid in saline. In the experiments on the delayed feedback mechanism steroids were injected in larger doses subcutaneously as suspensions in arachis oil 4 h before stress. The volumes injected were 0.25 ml/100 g body weight. The injections were given into an axilla, and when two injections were given to the same animals the second axilla was used to avoid leakage.

### *Estimation of corticosterone*

Corticosterone was measured fluorimetrically by a technique similar to that described by Zenker & Bernstein (1958) using a Hitachi Perkin-Elmer spectrophotofluorimeter.

In the experiments in which corticosterone was injected the basal plasma corticosterone concentration was elevated (see Table 1). In all experiments the magnitude of the response to the inhalation of ether was expressed as the difference in plasma corticosterone concentration or adrenal corticosterone production between stressed and unstressed rats treated with the same steroid (for basal values see Table 1). The significance of the difference in the values between groups of rats receiving different treatments was assessed by Student's *t*-test.

## Results

Inhalation of ether caused a rise in plasma corticosterone concentration from 138 ± 24 (s.e. mean) nmol/litre to 1032 ± 19 nmol/litre ($n = 100$) 25–30 min later, whilst *in vitro* corticosterone production rose from 25 ± 8 nmol 100 $mg^{-1}$ $h^{-1}$ to 161 ± 8 nmol 100 $mg^{-1}$ $h^{-1}$ ($n = 100$).

**Table 1** Plasma corticosterone concentrations (nmol/litre) in unstressed, vehicle-treated control and in steroid-treated rats killed either 40 min or 4.5 h after injection.

| *Treatment* | *Plasma corticosterone concentration (nmol/litre)* | |
|---|---|---|
| Vehicle control (saline) | 96 ± 19* | |
| (arachis oil) | | 141 ± 19 |
| | *100 µg steroid/100 g body wt. 40 min before death* | *2 mg steroid/100 g body wt. 4.5 h before death* |
| Pregnenolone | — | 130 ± 14 |
| 17α-Hydroxypregnenolone | — | 190 ± 30 |
| Progesterone | 116 ± 6 | 86 ± 22 |
| 11β-Hydroxyprogesterone | 88 ± 14 | 119 ± 14 |
| 11α-Hydroxyprogesterone | — | 104 ± 8 |
| 11β, 17α-Dihydroxyprogesterone | 75 ± 17 | 149 ± 22 |
| 17α-Hydroxyprogesterone | 121 ± 25 | 116 ± 19 |
| 11-Deoxycorticosterone | 110 ± 11 | 149 ± 14 |
| 18-Hydroxydeoxycorticosterone | 97 ± 14 | 135 ± 14 |
| Cortisol | 154 ± 19 | 122 ± 11 |
| Corticosterone | 518 ± 30* | 113 ± 22 |

Each result is the mean ± s.e. of the mean ($n = 6$).
Vehicle control *vs.* corticosterone: * $P < 0.001$.

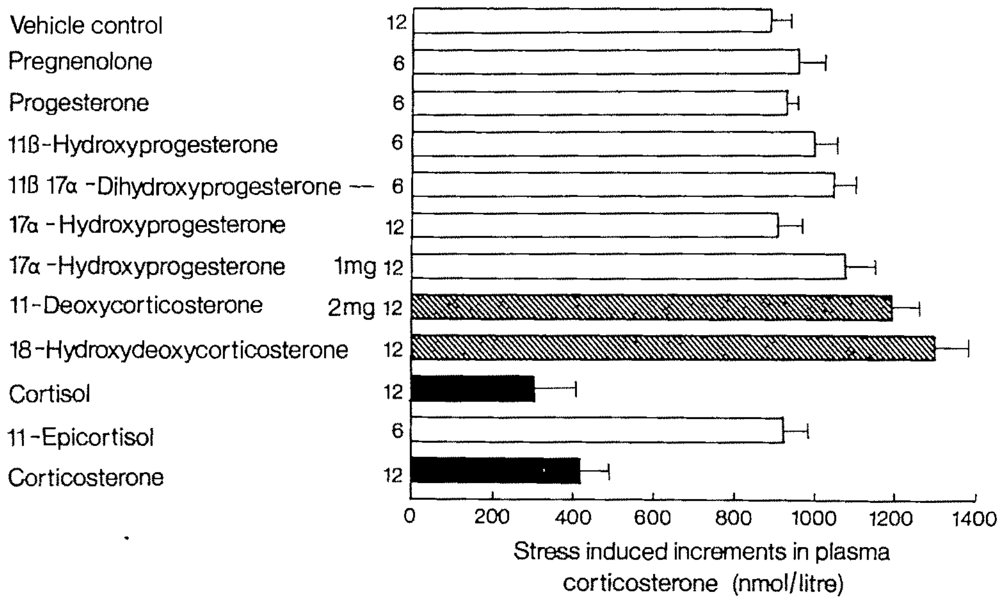

**Figure 1** Ether-induced increases in plasma corticosterone concentrations (nmol/litre) in rats injected (s.c.) with either saline (vehicle) or a steroid (100 µg/100 g body wt. unless otherwise stated) 10 min before ether inhalation and the plasma corticosterone measured 25–30 min after stress. Each column is the mean + s.e. mean of 6 to 12 animals. Solid columns show a significant reduction ($P < 0.001$) and hatched columns a significant increase in the reaction to ether inhalation ($P < 0.01$) when compared with the vehicle control.

Plasma corticosterone concentrations of unstressed animals injected subcutaneously with various steroids in a dose of either 100 µg or 2 mg/100 g body wt. are shown in Table 1. It can be seen that any possible conversions of these steroids to corticosterone were not reflected in plasma corticosterone concentrations.

### *Fast feedback*

The effect of various steroids in a dose of either 100 µg or 1–2 mg/100 g body wt. injected 10 min before stress upon the stress-induced increments in plasma corticosterone concentrations is shown in Figure 1. Corticosterone and cortisol in doses of 100 µg/100 g body wt. caused significant reductions in the stress response ($P < 0.001$), whereas 11-deoxycorticosterone (2 mg/100 g body wt.) and 18-hydroxydeoxycorticosterone (100 µg/100 g body wt.) caused significant exaggeration of the stress response ($P < 0.01$). 3β-Hydroxypregn-5-en-20-one (pregnenolone), pregn-4-ene-3, 20-dione (progesterone), 11β-hydroxyprogesterone, 11β, 17α-dihydroxyprogesterone, 17α-hydroxypregn-4-ene-3, 20-dione (17α-hydroxyprogesterone) and 11-epicortisol were found to exert no fast feedback effect. Similar results with these steroids were obtained when *in vitro* corticosterone production was used as the index of ACTH release.

The possibility that 18-hydroxydeoxycorticosterone pretreatment interfered with the fast feedback effect of corticosterone, causing a significant exaggeration of the corticotrophic response to stress was investigated (Figure 2). Corticosterone in a dose of 100 µg/100 g body wt. administered 10 min before stress reduced the corticotrophin release as assessed by the stress-induced increments in plasma corticosterone ($P < 0.001$) and the *in vitro* corticosterone production ($P < 0.01$). When 18-hydroxydeoxycorticosterone (100 µg/100 g body wt.) was given 10 min before the corticosterone the response of corticosterone production and release to ether stress was no longer inhibited by the corticosterone injection.

### *Delayed feedback*

The effects of various steroids injected 4 h before ether inhalation upon the stress-induced increments in plasma corticosterone are shown in Figure 3. The stress-induced increments in plasma corticosterone were reduced in rats injected with corticosterone, cortisol, 11-deoxycorticosterone, 11-deoxycortisol, 11β, 17α-dihydroxyprogesterone or 11β-hydroxyprogesterone 2 mg/100 g body wt. ($P < 0.01$). After the same dose of 17α-hydroxyprogesterone or 18-hydroxydeoxycorticosterone, however, there was exaggeration of the stress response at this time interval

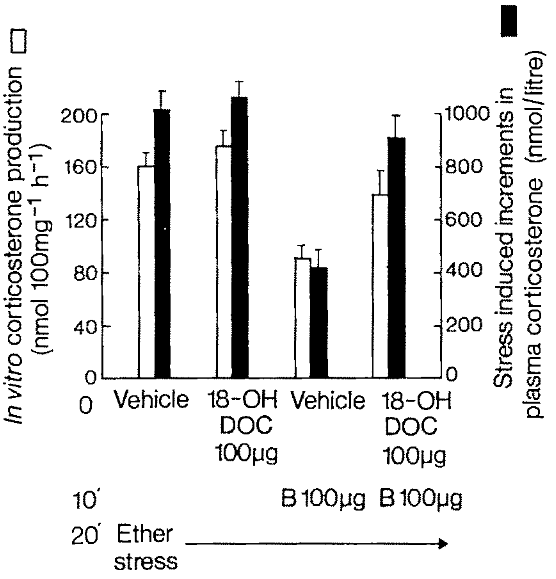

**Figure 2** Ether-induced increase in plasma corticosterone concentration (nmol/litre) and *in vitro* corticosterone production (nmol 100 $\mathrm{mg}^{-1}$ $\mathrm{h}^{-1}$) in rats injected (s.c.) at zero time with saline (vehicle) or 18-hydroxydeoxycorticosterone (18-OH DOC, 100 µg/100 g body wt.) and at 10 min with corticosterone (B, 100 µg/100 g body wt.). The animals were exposed to ether at 20 min and killed 25 to 30 min later. Each column is the mean+s.e. of the mean of 6 animals. Steroid secretion in the 18-OH DOC+B-treated rats was significantly higher than in vehicle+B-treated rats ($P<0.01$).

($P<0.01$). Similar results were obtained using *in vitro* corticosterone production.

The possibility that 18-hydroxydeoxycorticosterone or 17α-hydroxyprogesterone administered some hours before stress altered corticosterone-induced fast feedback was investigated (Table 2, experiment 1). Corticosterone injected in a dose of 100 µg/100 g body wt. 10 min before stress reduced significantly the stress-induced increments in plasma corticosterone and the *in vitro* corticosterone production compared to vehicle control ($P<0.05$). 18-Hydroxydeoxycorticosterone or 17α-hydroxyprogesterone (2 mg/100 g body wt.) given 4 h before the corticosterone reduced the inhibitory action of corticosterone on the response to stress applied 10 min after the corticosterone administration.

Finally the possibility that 18-hydroxydeoxycorticosterone or 17α-hydroxyprogesterone administered several hours before stress may alter corticosterone-induced delayed feedback activity was investigated (Table 2, experiments 2 and 3). Experiment 2 shows the effect of combining a dose of 18-hydroxydeoxycorticosterone (2 mg/100 g body wt. which was capable of exaggerating stress-induced ACTH release) administered 2 h beforehand, with an inhibitory dose of corticosterone (400 µg/100 g body wt.). There was a reduced response to stress applied 4 h after the first injection as assessed by both indices, indicating that 18-hydroxydeoxycorticosterone did not interfere with the delayed feedback action of corticosterone. Neither did 17α-hydroxyprogesterone (experiment 3).

## Discussion

It is evident from the results in Table 1 that the steroids tested do not act at the adrenal level by becoming involved in the biosynthetic pathway or by substrate inhibition (see also: Kraulis, Traikov, Li & Birmingham, 1973). Furthermore, Kraulis *et al.* (1973) found that adrenal glands removed from rats treated with 11β-hydroxyprogesterone, 11-deoxycorticosterone, 18-hydroxydeoxycorticosterone or corticosterone (1 mg/100 g body wt. for 3 days) responded to ACTH stimulation *in vitro* with a similar increase in *in vitro* corticosterone production to that in control animals. More important, therefore, is the conversion or metabolism of steroids at the hypothalamic and pituitary levels. The dependence of the feedback response on conversion of steroids in other parts of the body (e.g. liver) has been excluded as these steroids have a similar activity *in vitro* on CRF release when added to the hypothalamus *in vitro* (Jones, Hillhouse & Burden, 1976).

The proposed theory that two different receptors are involved in the fast and delayed corticosteroid feedback control of ACTH secretion (Jones *et al.*, 1974) is supported by the experiments described here. The fast feedback receptor mechanism appears to be highly specific for corticosterone and cortisol, requiring the 11β-hydroxyl group (as 11-epicortisol is not active) and the 21-hydroxyl group (as progesterone and 11β-hydroxyprogesterone are inactive). Interestingly, replacing the 11β-hydroxyl group of corticosterone and cortisol by a hydrogen atom increases the affinity but not the efficacy, and the corresponding steroids are antagonists to corticosterone and cortisol (Jones *et al.*, 1974). The delayed feedback receptor mechanism appears to be less specific since corticosterone, cortisol, 11-deoxycorticosterone, 11-deoxycortisol, 11β-hydroxyprogesterone and 11β-, 17α-dihydroxyprogesterone all exert a feedback action. The presence of either the 11β-hydroxyl group (as 11β-hydroxyprogesterone is effective) or the 21-hydroxyl group (as 11-deoxycorticosterone is effective) appears to be necessary for delayed feedback activity. Again the 11α-hydroxyl group renders the steroid inactive, as in the case of 11-epicortisol and 11α-hydroxypregn-4-ene-3, 20-dione (11α-hydroxyprogesterone).

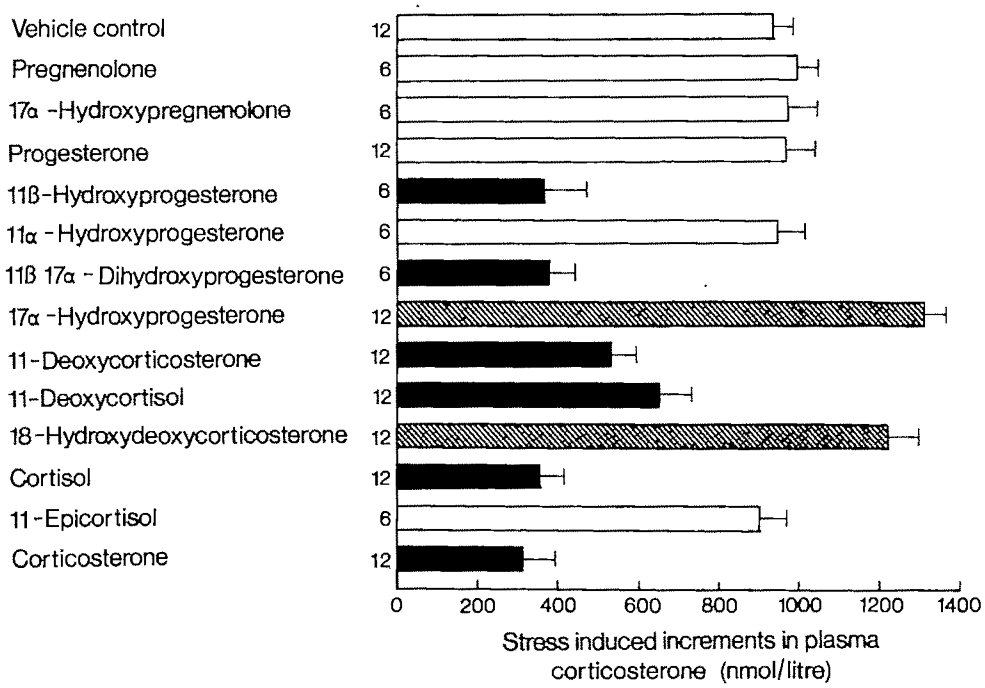

**Figure 3** Ether-induced increases in plasma corticosterone concentration (nmol/litre) in rats injected (s.c.) with either arachis oil (vehicle) or a steroid (2 mg/100 g body wt.) 4 h before stress. Each column is the mean + s.e. of the mean of 6 to 12 animals. Solid columns are significantly lower ($P < 0.01$, or less) and hatched columns are significantly higher ($P < 0.01$) than the vehicle control.

**Table 2** Ether-induced increments in plasma corticosterone concentration (nmol/litre) and in the *in vitro* corticosterone production (nmol 100 mg$^{-1}$ h$^{-1}$) in rats injected (s.c.) with either arachis oil, 18-hydroxydeoxycorticosterone (18-OH DOC), 17α-hydroxyprogesterone (17α-OH Prog), corticosterone (B) or a combination of these steroids at different time intervals before ether inhalation.

| *Treatment* | | *Dose/100 g body wt.* | *Ether-induced increments in plasma B (nmol/litre)* | In vitro *B production (nmol 100 mg*$^{-1}$h$^{-1}$) |
|---|---|---|---|---|
| *Expt. 0:* | Vehicle control | | $1021 \pm 58^{**}$ | $152 \pm 14^{*}$ |
| | 18-OH DOC | 2 mg | $1223 \pm 66$ | $240 \pm 14$ |
| | 17α-OH Prog | 2 mg | — | $226 \pm 8$ |
| *Expt. 1:* | Vehicle + B | 100 µg | $588 \pm 97^{**}$ $^{**}$ | $97 \pm 14^{*}$ $^{*}$ $^{***}$ |
| | 18-OH DOC + B | 2 mg + 100 µg | $999 \pm 69$ $^{**}$ | $174 \pm 25$ $^{*}$ |
| | 17α-OH Prog + B | 2 mg + 100 µg | — | $177 \pm 8$ $^{***}$ |
| *Expt. 2:* | Vehicle + B | 400 µg | $566 \pm 91$ | $108 \pm 22$ |
| | 18-OH DOC + B | 2 mg + 400 µg | $765 \pm 99$ | $105 \pm 25$ |
| *Expt. 3:* | Vehicle + B | 400 µg | — | $50 \pm 22$ |
| | 17$^{+}$-OH Prog + B | 2 mg + 400 µg | — | $69 \pm 17$ |

In Expt. 1 the steroids were injected 3 h 50 min apart, in Expt. 2, 2 h apart and in Expt. 3, 1 h apart. All groups of animals were stressed 4 h after the initial injection. Each result is the mean ± s.e. mean from six animals. The position of the asterisks to the right of each column of figures indicate which experimental values are being compared.

Student's $t$-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

The fact that such a wide variety of steroids are agonists on the delayed feedback mechanism is surprising, particularly since many of these steroids have little glucocorticoid or anti-inflammatory activity. This reinforces our previous suggestion that an analysis of the structure-activity relationship may enable the design of a steroid with strong anti-inflammatory activity and little feedback suppression, or a steroid that specifically antagonizes the feedback action of potent anti-inflammatory steroids (Jones *et al.*, 1974). An antagonist to the delayed feedback action of corticosteroids would be the most useful since the latter must be the main mechanism of the unwanted ACTH suppression following therapeutic use of steroids.

The rat adrenal *in vitro* has the capacity to 17$\alpha$-hydroxylate progesterone, but this system is inhibited in the presence of the 11$\beta$-hydroxylating system (Young & Sweat, 1967). However, cortisol and 17$\alpha$-hydroxyprogesterone are not found in the adrenal vein blood of the rat, although they have been shown in this study to have interesting feedback actions. The steroid 18-hydroxydeoxycorticosterone however, is an adrenal steroid in the rat (Birmingham & Ward, 1961; Péron, 1961) and its secretion is increased by ACTH (Ward & Birmingham, 1960). This steroid has actions on electrolyte and water excretion in this species (Birmingham, MacDonald & Rochefort, 1968) and it has been implicated as a possible hypertensive agent in rats (Birmingham, Rochefort & Traikov, 1965; Rapp & Dahl, 1971) and in man (Melby, Dale & Wilson, 1971). In a recent study, unilaterally nephrectomized male rats maintained on 1% saline were injected with vehicle, corticosterone, 11-deoxycorticosterone or 18-hydroxydeoxycorticosterone 200 µg/daily for 21 days (Oliver, Birmingham, Bartova, Li & Chan, 1973). Oliver *et al.* (1973) showed that not only 11-deoxycorticosterone but 18-hydroxydeoxycorticosterone contributes to the aetiology of hypertension possibly by a mechanism involving stress-induced ACTH release. The same laboratory had previously shown that rats treated for 3 days with 18-hydroxydeoxycorticosterone (1 mg/100 g body wt. twice a day) responded on the third day with abnormally high plasma corticosterone concentrations and *in vitro* corticosterone production in response to ether stress (Kraulis *et al.*, 1973). Our experiments confirm that 18-hydroxydeoxycorticosterone is capable of exaggerating stress-induced ACTH release, and this is probably due to an interference with the fast feedback mechanism of corticosterone. 18-Hydroxydeoxycorticosterone may therefore play a role influencing ACTH secretion in the rat and other species.

The current work and that reported previously (Jones *et al.*, 1974) reinforce our contention that high circulating levels of adrenal corticosteroid precursors found in adrenogenital syndrome (11-deoxycortisol, 11-deoxycorticosterone and 17$\alpha$-hydroxyprogesterone) antagonize the fast feedback action of cortisol. This antagonism might provide another mechanism whereby ACTH levels are elevated in this disease, and explain why treatment with physiological doses of glucocorticoids often fails to reduce the high circulating levels of ACTH (Jacobs, Abraham, Glasser, Hopper & Kondon, 1972). The high levels of 17$\alpha$-hydroxyprogesterone at mid cycle during the human menstrual cycle may also account for the elevated ACTH levels at this time (Burns, 1975).

We wish to thank Organon Laboratories Ltd. for generous gifts of corticosteroids and Mr C.E. Horth of G.D. Searle & Co. for the generous gift of 18-hydroxydeoxycorticosterone. This research was supported by a grant (to M.T.J.) from the Wellcome Trust.

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*(Received March 24, 1976. Revised June 30, 1976.)*

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# ATROPINE RESISTANCE AND MUSCARINIC RECEPTORS IN THE RAT URINARY BLADDER

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**1** The action of an anticholinesterase and an antimuscarinic drug upon nerve-induced contractions of the rat urinary bladder were examined during transmural stimulation at 20 Hz. Responses were graded in magnitude by limiting the duration of the stimulus trains.

**2** Responses of low magnitude produced by short stimulus trains were unchanged by atropine; however, maximal responses resulting from long stimulus trains were diminished in magnitude and shortened in duration.

**3** Responses of small magnitude elicited by short stimulus trains involve muscarinic receptors in close proximity to the neuroeffector junction and are resistant to atropine.

**4** Maximal responses elicited by long stimulus trains involve 'junctional' muscarinic receptors as well as receptors located at the periphery of the junction; the 'extrajunctional' receptors are blocked by atropine.

**5** Responses of low magnitude produced by short stimulus trains were unaffected by echothiophate; however, the duration of maximal responses resulting from the long stimulus trains was extended.

**6** The inhibition of cholinesterase did not increase the occupation of muscarinic receptors by the transmitter; however, after large quantities of transmitter were released by the long stimulus trains the association between the receptors and acetylcholine was prolonged.

## Introduction

The motor innervation of the mammalian urinary bladder is usually represented as cholinergic even though nerve-induced responses of this organ are not completely prevented by conventional antimuscarinic agents such as atropine or scopolamine (Ursillo & Clark, 1956; Carpenter, 1963; Vanov, 1965; Ambache & Zar, 1970). Although these drugs do reduce or block bladder contractions elicited by carbachol or by acetylcholine itself, some authors have proposed that the motor nerve terminals of the urinary bladder are non-cholinergic (Ambache & Zar, 1970; Dumsday, 1971; Burnstock, Dumsaday & Smythe, 1972). The atropine resistance of the urinary bladder may result from a close association between the nerve terminals of this organ and the muscle cell membrane; the junction or point of contact between the post-ganglionic nerves and the smooth muscle membrane may not be readily accessible to antimuscarinic agents.

There is strong evidence that acetylcholine is a transmitter in the rat urinary bladder: (1) nerve-induced responses of the bladder are potentiated by physostigmine (Ursillo & Clark, 1956; Carpenter, 1963; Chesler & Thorp, 1965; Huković, Rand & Vanov, 1965) (2) acetylcholine accumulates in the bathing solution *in vitro* in proportion to the stimulus rate (Carpenter & Rand, 1965; Chesler, 1967); (3) the responses of the rat bladder and the release of acetylcholine during stimulation are greatly reduced following pretreatment of the rats with *Cl. botulinum* toxin (Carpenter, 1967).

This study is concerned with the action of ecothiophate and atropine on nerve-induced responses of the urinary bladder *in vitro*. Its purpose is to show that those junctional events elicited by the small amount of transmitter released during brief stimulation were not affected by substances which block muscarinic receptors or by substances which extend the life of the transmitter. However, during prolonged stimulation which elicits more powerful contractions of the muscle, events at the junction were very significantly affected by these drugs; the contractile responses were more susceptible to the blocking actions of an antimuscarinic agent such as atropine and to a drug such as echothiophate which prolongs the action of the transmitter.

## Methods

Phasic contractions of the rat bladder were elicited isometrically in a constant temperature water bath

(Carpenter, 1963). The organ was stimulated by single or repetitive electrical pulses of 1 ms duration. A constant stimulating current of 25 mA was passed across the bladder wall between two coaxial electrodes composed of platinum wire. One electrode located in the bladder lumen projected through the cannula on which the organ itself was secured. The other electrode was fixed in the organ bath and surrounded the preparation in the bathing solution. The cannula was connected to a pressure transducer by flexible tubing. Care was taken to displace any air bubbles before the experiment started. The magnitude of a given response was obtained from recordings of the pressure that developed while the stimuli were applied. Each response was recorded on an ink-writing oscillograph and was photographed on high speed film from a cathode ray oscilloscope (CRO).

The excitation of bladder smooth muscle by transmural stimuli is indirect and mediated by acetylcholine (ACh) released from postganglionic nerve (Carpenter & Rand, 1965); hexamethonium or mecamylamine did not affect the responses while tetrodotoxin and certain other local anaesthetics prevented them.

Throughout the experiments the stimuli were applied as single pulses or as trains of pulses at a rate of 20 Hz. Responses of four different magnitudes between 20 and 160 cmH$_2$O were produced by controlling the number of pulses; the mechanical response of a bladder preparation was graded by limiting the duration of the trains to 0.25, 0.5 and 5.0 seconds. The stimulus trains were applied intermittently and it was found that a 3 min period was adequate for recovery as shown by the consistent reproducibility of the responses for as long as 90 minutes.

There are several mechanical factors which can influence the contractility of the rat bladder. Both the velocity and the magnitude of a response are related to the initial length of the muscle fibres and to their state of elasticity. Throughout its physiological capacity as a storage organ the muscle in the rat bladder displays a length-tension relation similar to cardiac or somatic muscle. In these experiments the volume in the organ was maintained at 0.15 ml which is much less than the volume it contains at the time of voiding. Nevertheless, under these conditions the muscle fibres develop their maximal force during an isometric contraction; this indeed suggests that the muscle fibres were set at their optimal length. Moreover, since the volume was constant during a contraction, the force developed by the response can be calculated from the Laplace relation: $T = P.r^2$, where T is the force or tension, P is the pressure and r is the radius of the bladder which may be assumed to be spherical.

Echothiophate (0.3 mg/kg) or atropine sulphate (2.5 mg/kg) were administered to adult male rats weighing between 400 and 450 grams. Within 30 min the animals were killed, the bladder removed and placed in a bathing solution of the following composition (mM): NaCl 145, KCl 2.7, CaCl$_2$ 2, MgCl$_2$ 0.25, MOPS (morpholino-propane-sulphonic acid) buffer adjusted to pH 7.3, 10 and glucose 10. The dose of echothiophate that was administered amounted to 10 times the rabbit LD$_{50}$ (Koelle & Steiner, 1956) and its action on the bladder did not diminish while the experiments were carried out. On the other hand, bladder preparations taken from the atropine-treated animals were suspended in the bathing solution containing atropine sulphate 20 μM.

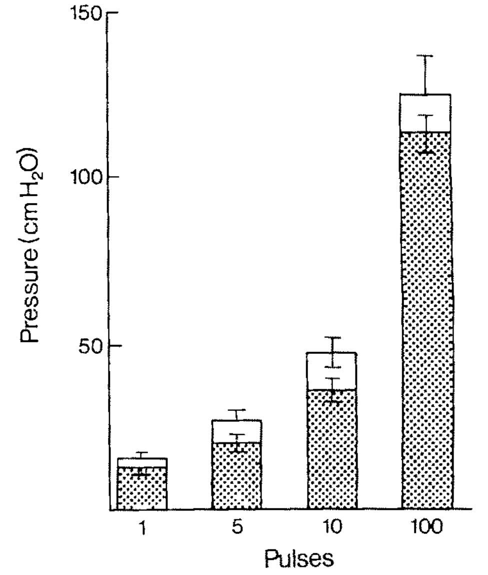

**Figure 1** The pressure developed by rat urinary bladder *in vitro* during stimulation by electric pulses. The mean response to a single stimulus is shown by the 1st column and the mean responses to 5, 10 and 100 pulses applied at a stimulus rate of 20 Hz are shown by the 2nd, 3rd and 4th columns respectively. Ordinate scale: pressure in cmH$_2$O. The number of pulses that were applied appears at the base of each column. Preparations taken from untreated control rats are represented by the open area ($n = 12$) while the preparations removed from echothiophate iodide-treated (0.3 mg/kg) rats ($n = 12$) are represented by the stippled area. The s.e. is indicated by the vertical lines.

## Results

### *Bladder contraction in control preparations produced by single stimulus pulses*

Phasic contractions of the rat bladder produced by single stimuli developed only 1/10 as much force as

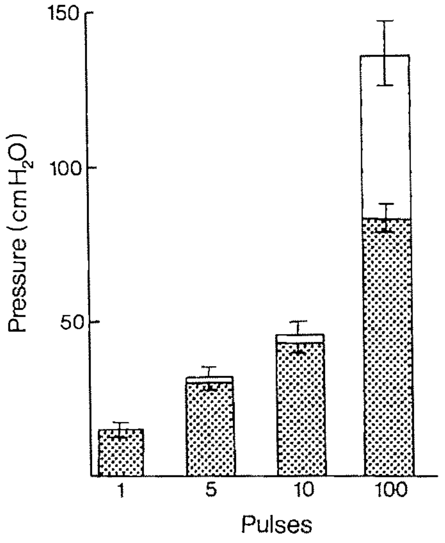

**Figure 2** The pressure developed by the rat urinary bladder *in vitro*. Preparations taken from untreated control rats are represented by the open area ($n=13$) while the preparations removed from atropine-treated (2.0 mg/kg) rats ($n=10$) are represented by the stippled area. The s.e. is indicated by the vertical lines. Other details as for Figure 1.

those elicited by the 20 Hz 5 s stimulus trains. After only one pulse was applied to the coaxial electrodes, contraction between 12 and 18 cmH$_2$0 were produced (Figures 1 and 2). In 15 observations the mean pressure developed by the preparations amounted to 16 ± 2. Within 0.2 s (latency) after the pulse was applied the peak pressure developed some 2 s later and subsided to the original resting pressure in approximately 8 s (relaxation time).

### *Bladder contractions produced by dual stimulus pulses*

When a second pulse was applied after the initial pulse a further increment of pressure was produced by the bladder muscle. This increment is expressed in Figure 3, as the mean of the ratios of the response following two pulses to the response after only pulse. If the ratio is greater than one, the contraction elicited by a second stimulus summated mechanically with the initial contraction. The time course of summation was examined at intervals of 0.1, 1.0, 5, 10 and 20 s, and appear as open circles in Figure 3. It will be noted that the response to the second pulse declined progressively when the interval was longer than 5 seconds. Nevertheless, since the ratio at 10 s was greater than one, summation could occur at this interval. However, at 20 s the ratio was always less than one.

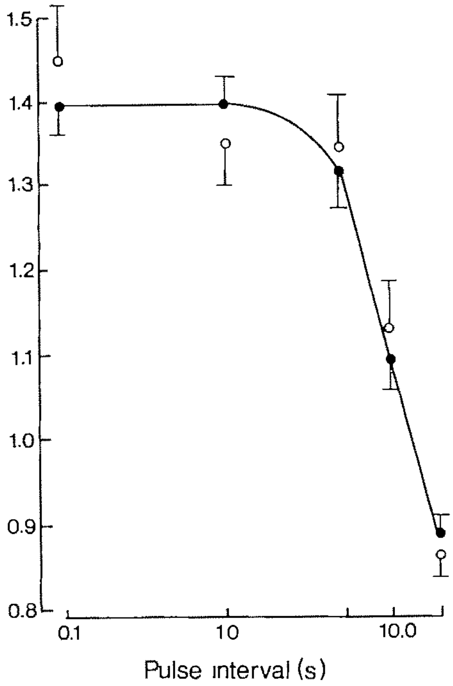

**Figure 3** The relative magnitude of bladder responses elicited by 2 stimulus pulses applied at different intervals. Ordinate scale: response to dual pulses/responses to single pulse, expressed as the mean. Abscissa scale: interval between pulses in seconds. (○) Preparations taken from untreated rats ($n=6$); (●) preparations taken from rats treated with 0.3 mg/kg echothiophate iodide ($n=5$). The s.e. is indicated by the vertical lines.

### *Bladder contractions after single and dual stimuli of preparation taken from echothiophate-treated animals*

In the group that had been given the cholinesterase inhibitor and had been stimulated by single pulses, the latency, the response time, the relaxation time and the mean of the responses themselves were not different from the control group. In Figure 1 the mean response of the treated group is shown by the shaded area in the first column.

If phasic contractions of the bladder are mediated by ACh, the transmitter released during a response would most probably be hydrolyzed prior to relaxation. Indeed, if acetylcholinesterase restricts the accumulation of the transmitter, the response of the treated preparations to dual stimuli would be different from the control; if the action of the transmitter which had been released after the first stimulus had been prolonged by echothiophate the responses elicited by the second stimulus would have been displaced above the control group. However, the data obtained from

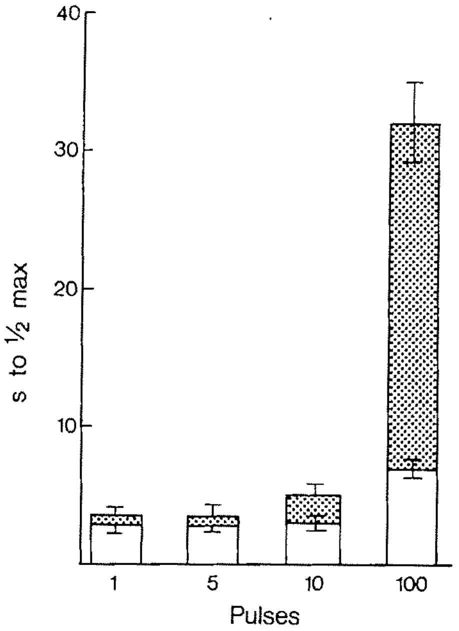

**Figure 4** The duration of contractile responses of the rat urinary bladder elicited by a single stimulus pulse (1st column) and by stimulus trains of 5, 10 and 100 pulses (2nd, 3rd and 4th columns). The mean of 8 non-treated control animals is represented by the open area of each column while the 10 rats treated with echothiophate iodide (0.3 mg/kg) is shown by the stippled area. The s.e. is indicated by the vertical lines.

control and treated groups shown in Figure 3 are not significantly different.

### *Bladder contractions produced by stimulus trains*

Repetitive stimulation of the bladder elicits responses which depend upon the stimulating frequency and the number of stimuli that are applied. In the range of 1 to 20 Hz the magnitude of a contraction is related to the stimulus rate. At 20 Hz, around 100 pulses are usually necessary for the bladder to produce a maximal response. The mean responses of the two groups, shown in the last column of Figures 1 and 2, amounted to 125 ± 12 and 136 ± 10 $cmH_2O$ respectively. The third columns of Figures 1 and 2 indicate the mean responses which were produced when only 10 pulses were applied and amounted to roughly one-third of maximal or 47 ± 5 and 46 ± 3 $cmH_2O$ respectively. After 5 stimulus pulses were applied the mean contractile response of the two groups, which appear in the second columns, were 37 ± 1 and 32 ± 1 $cmH_2O$.

### *Bladder contractions produced by stimulus trains in treated preparations*

*Echothiophate.* Echothiophate did not increase the magnitude of the contractile responses that resulted from multiple stimuli. In fact, the stimulus trains elicited somewhat less of a pressure rise in the treated group than in the untreated controls. In Figure 1 the mean responses, shown in the second and third column and elicited by 5 and 10 stimulus pulses respectively, were significantly less in the group treated with echothiophate. Although the mean of the responses elicited by the 5 s stimulus train was less than control, the difference between control and treated groups was not significant (fourth column, Figure 1).

Despite the failure of echothiophate to enhance contractile responses to single, dual or to trains of stimuli, acetylcholinesterase nevertheless, does appear to limit the transmitter action of acetylcholine in the bladder. The duration of a response following single stimuli and following the three stimulus trains was measured for control and the echothiophate-treated groups, In Figure 4 this is expressed as the average number of seconds required for the pressure to diminish to one-half the value of the peak response (relaxation time). After single stimuli and after trains of 0.25 and 0.5 s, relaxation occurred within 3 s in the control untreated group; the height of the first three columns is the same. After the 5 s trains the mean relaxation time was 7 seconds. There is little difference between treated and control groups in the first three columns; the mean relaxation time following short periods of stimulation was not significantly prolonged by the inhibitor. However, the relaxation time following the 5 s stimulus train was extended by 5-fold from control to nearly 35 (last column, Figure 4).

Recordings of the contractile responses by a control bladder preparation to the various stimulus trains are shown in Figure 5. It should be compared with the recordings of the responses to the same stimulus trains of a treated preparation which is shown in Figure 6. These records indicate further, that the most significant prolongation of the responses occurred following the longest stimulus train. Only in the latter instance was the response time of the treated preparation significantly extended from the control.

*Atropine.* Atropine (20 μM) did not exert a significant action on the rat bladder if the responses were elicited by the short stimulus trains. In the third column of Figure 2, the 20 Hz stimulus train was applied for only 0.5 s; the mean response of the control and of the treated group were not significantly different. The mean responses shown in the first and second column also show that a bladder contraction resulting from either a single pulse or a train applied for only 0.25 s was not changed by atropine. Accordingly, any response elicited by one, five or as

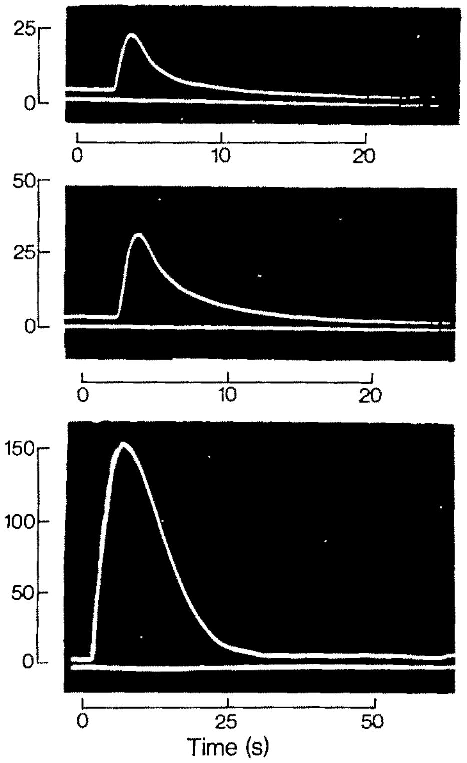

**Figure 5** Recordings taken from a CRO of the contractile responses of a control untreated rat urinary bladder after 20 Hz stimulus trains were applied for 0.25 (upper), 0.5 (middle), and 5.0 s (lower). The upper and middle traces were made at twice the gain and 2.5 times the sweep speed as the lower trace. The appropriate pressure calibrations appear in the left margins while the time base is located below the recordings.

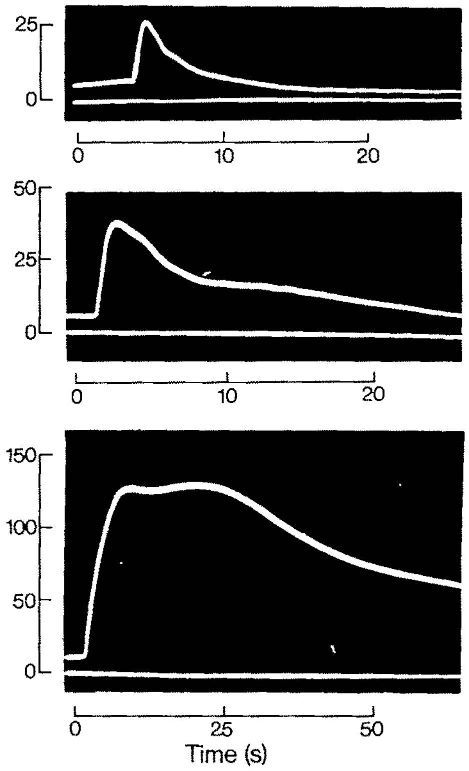

**Figure 6** Recordings taken from a CRO of the contractile responses of a bladder preparation removed from a rat treated with echothiophate (0.3 mg/kg). Stimulus trains at 20 Hz were applied for 0.25 (upper), 0.5 (middle), and 5.0 s (lower). The upper and middle traces were made at twice the gain and 2.5 times the sweep speed as the lower trace. The appropriate pressure calibrations appear in the left margins while the time base is located below the recordings.

many as 10 pulses was not changed significantly by this drug even though it completely prevented the action of exogenous ACh (50 μM) and carbachol (25 μM). However, there was one further effect caused by the atropine which suggested that there was a partial blockade of muscarinic receptors which were involved in the nerve-induced responses of the control preparations. This was evident by the very significant difference (40%) between the mean responses of the control and the atropine-treated preparations resulting from stimulation by the 5 s stimulus trains (last column, Figure 2).

The contractile force of the rat bladder developed quite rapidly during repetitive stimulation at 20 Hz and the intraluminal pressure continued to increase throughout the 5 s stimulus train (Figure 7). Atropine (20 μM) did not interfere with the response which developed initially; the rate at which the pressure developed during the first two seconds appeared to be identical in the control and in the treated animals. In Figure 7 are shown two recordings of the contractile response elicited by a 5 s, 20 Hz train; the upper trace in each case was obtained from a control preparation; the lower trace was the result of the treatment with atropine. In 25 experiments the mean response of a non-treated preparation was always greater than the response following atropine. Nevertheless, the rising phase of the contractions; which of course represented the initial response to the stimulus train, was the same in both cases; treated bladders maintained the same rate of contraction during the initial 0.5 s of the response as the controls. In a number of others the initial rise time appeared to be unchanged from the control for as long as one second. The blocking action

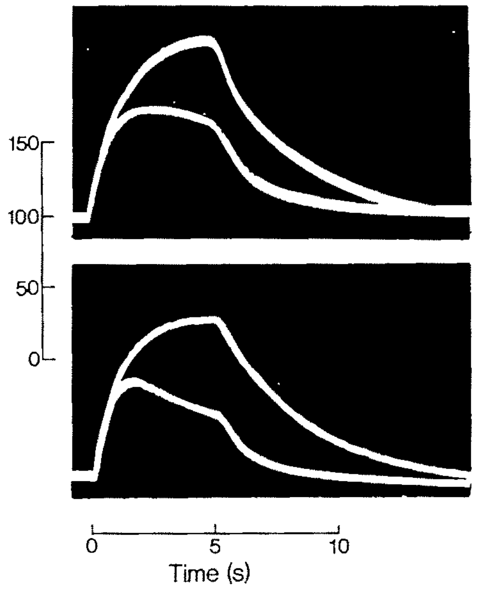

**Figure 7** Recordings taken from a CRO of the contractile responses of the bladder from two rats, showing the response of the control untreated rat urinary bladders (upper trace in each case) and response after they were treated with atropine sulphate (20 µM) (lower trace). The pressure calibration appears in the left margins and the time base is located below the recordings.

of atropine was only manifested later during the peak of the contraction in treated preparations. Indeed, the most striking effect of the atropine appeared during the final 3 s of the response itself; the muscle appeared to be undergoing relaxation during the latter part of the stimulation period (Figure 7). In contrast, the control preparations either maintained a constant pressure throughout the stimulation period or would continue to rise significantly while the stimulus train was being applied.

## Discussion

During a contractile response the pressure in the bladder is elevated in proportion to the number of receptors occupied by the transmitter. Transmural stimulation in the range 1–20 Hz elicits responses in proportion to both stimulus rate and the amount of transmitter that is released during the stimulus period. When stimuli are applied at 20 Hz for a 5 s period much more of the transmitter diffuses from the junction than would occur after single, dual or short trains of stimuli. Accordingly, there would be a greater involvement of muscarinic receptors that are located some distance from the junction since the actual contact between the nerve terminal and the muscle fibres themselves is probably quite small.

### *Echothiophate and receptor-transmitter combination*

Acetylcholinesterase apparently plays no major role in limiting the combination of transmitter with the muscarinic receptors; there was no difference between the echothiophate-treated and the control groups with respect to the peak pressure responses, as shown in Figure 1. The difference between these two groups was in the duration of the responses following the long stimulus trains. It would thus appear that transmitter action on some but not all receptors was prolonged by AChE inhibition. Since the response to single, dual (Figure 3) and the short stimulus trains was not affected by echothiophate, while maximal responses were prolonged by it (Figure 4 and 6), it seems reasonable to suppose that echothiophate either cannot gain access to the space close to the junction or that hydrolysis of the transmitter occurs only at some distance from the junction.

### *Atropine and receptor-transmitter combination*

The recordings of Figure 7 indicate that the diffusion of ACh from the junction during a long stimulus train must be quite extensive; the recruitment of all muscarinic receptors was completed after 5 s of stimulation. However, much less time was needed for recruitment during single, dual and the short stimulus trains. In the latter cases receptor-transmitter combination may have occurred much more rapidly with receptors close to the junction. Of course during the diffusion of transmitter from the junction the concentration of transmitter would decrease exponentially with distance.

Atropine was not an effective antagonist of the transmitter when single, dual or short stimulus trains were employed (Figure 2). If the concentration of ACh is very high in the junctional area the receptors would be more resistant to blockade by atropine. Moreover, the early phase of the response shown in Figure 7 was not impaired by atropine. Only during the late phase of the response was any antagonism between atropine and the transmitter clearly demonstrated. The late phase of a prolonged response would involve more distant receptors. These 'extrajunctional receptors' would be exposed to lower concentrations of transmitter as would be anticipated by diffusion and they would be more vulnerable to blockade. Phasic contractions of the guinea-pig bladder elicited by 10 Hz trains of 2–5 pulses were similarly resistant to large doses of atropine (Ambache & Zar, 1970) while contractions produced by single pulses were not enhanced by physostigmine. Accordingly, in this species as well, anticholinesterase and antimuscarinic agents were without effect on bladder responses of small magnitudes.

### *Junctional and extrajunctional receptors in the rat bladder*

There are major differences between a response produced *in vitro* by a cholinomimetic such as carbachol or acetylcholine and a response elicited by stimulation with electric pulses. Atropine, at a concentration of 1 µM for example, will compete with the agonists in the bathing solution causing a shift in the dose-response curve. A larger concentration of atropine, 20 µM, will eliminate the response to the cholinomimetics altogether. In this concentration atropine will block only 40% of the bladder response to the long stimulus trains. However, a maximal response which results from the long stimulus train is some 50% higher than the maximal response to high concentrations of the cholinimimetics (Carpenter & Rand, 1965; Carpenter, 1967). This suggests that the entire population of muscarinic receptors cannot combine with the cholinomimetic in the bathing solution; the junctional receptors may not combine with the agonist because of limitations of access. The difference between the neuroeffector junction of the bladder and other cholinergic junctions with respect to 'atropine resistance' may be partially resolved by the division of the receptor population into two spatially distinct sites.

### *Non-cholinergic transmission in the rat urinary bladder*

These results offer no support for a non-cholinergic transmitter in the rat urinary bladder. Echothiophate and atropine modified the responses of this organ in a way that could be predicted by the specificity of echothiophate for the cholinesterase enzyme and the specificity of atropine for muscarinic receptors. These actions of echothiophate and of atropine were manifested only during maximal responses. Under these conditions transmission would be accompanied by the release of a larger amount of transmitter than during stimulation by single, dual or short trains of stimuli. If two transmitters were involved a small submaximal response elicited by a few impulses would have to be mediated by a non-cholinergic transmitter while the maximal responses elicited by the long stimulus trains would somehow have to involve acetylcholine.

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*(Received April 6, 1976.)*

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# SELECTIVE RESERPINE-INDUCED SUPERSENSITIVITY OF THE POSITIVE INOTROPIC AND CHRONOTROPIC RESPONSES TO ISOPRENALINE AND SALBUTAMOL IN GUINEA-PIG ISOLATED ATRIA

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1 Dose-response curves for the positive inotropic and chronotropic responses to isoprenaline were obtained in atria from untreated guinea-pigs and those receiving various reserpine pretreatments.

2 Tension responses were unaffected, whereas rate responses were depressed by the lowest dose of reserpine (0.05 mg/kg i.p. at 24 hours).

3 With larger 24 h doses and a 3 day pretreatment, the rate and tension dose-response curves were progressively displaced to the left, indicating supersensitivity which was greater for tension at each pretreatment.

4 No supersensitivity to histamine or $Ca^{2+}$ could be detected, leading to the conclusion that it was selective for the $\beta$-adrenoceptor agonists possibly at the receptor level.

5 As an indication of the adrenergic neurone depleting effectiveness of each reserpine dosage, preparations were exposed to test doses of $\beta$-phenylethylamine.

6 Salbutamol was a partial agonist in untreated atria, the maximum rate (63.3%) and tension (10.0%) responses being less than those for isoprenaline. In atria from reserpine pretreated animals the supersensitivity was revealed as an increase of this maximum compared with isoprenaline.

7 The significance of this observation in relation to the possible mechanism of the supersensitivity is discussed.

## Introduction

The adrenergic neurone-depleting activity of reserpine is accompanied by a gradual increase in sensitivity of the effector organ to catecholamines (Fleming & Trendelenburg, 1961). This supersensitivity has been compared with that following decentralization of the organ since they have been found to exhibit similar characteristics of a slow onset of action and relative non-specificity (Trendelenburg, 1963). There are however many conflicting reports of failure to induce supersensitivity by reserpine, particularly in isolated tissues taken from pretreated animals. This is illustrated by the nictitating membrane, for which there are numerous demonstrations of supersensitivity in the intact animal (Trendelenburg & Weiner, 1962; Fleming, 1963; Seidehamel, Patil, Tye & LaPidus, 1966). Isolated preparations however have been shown to lack the enhanced sensitivity (Tsai, Denham & McGrath, 1968). Similarly cardiac muscle has shown discrepancies between intact and isolated hearts. Supersensitivity to the positive chronotropic effects of noradrenaline has been found in open-chest dogs (Trendelenburg & Gravenstein, 1958), and similar reports have been made for dog heart-lung preparations (Bejrablaya, Burn & Walker, 1958; Westfall & Fleming, 1968a) and guinea-pig hearts *in vivo* (Westfall & Fleming, 1968b). Potentiation of the rate responses to noradrenaline by reserpine-treatment has been shown in perfused hearts (Westfall & Fleming, 1968b), but not in guinea-pig isolated atria (Crout, Muskus & Trendelenburg, 1962; Westfall & Fleming, 1968b). The latter authors, like Tsai *et al.* (1968) for the nictitating membrane, attributed this failure to demonstrate supersensitivity to the degree of trauma on removal from the animal.

The positive inotropic responses to norádrenline have received less attention, being studied exclusively in isolated perfused hearts where McNeill & Schulze (1972) have reported satisfactory sensitization.

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Taylor, Westfall & Fleming (1974) on the other hand, claim that it is only the chronotropic effects that are enhanced by reserpine, and any potentiation of the inotropic responses is due to the influence of the rate changes upon the tension in spontaneously beating hearts.

In view of these conflicting reports of supersensitivity between the *in vivo* and *in vitro* hearts and the possible differences between the positive inotropic and chronotropic responses to catecholamines, we have examined the effects of reserpine pretreatment upon the responses to isoprenaline in our experimental model. This consists of separated left and right guinea-pig atria; the spontaneously beating right atrium provides the rate responses and the left atrium is driven at a constant rate to provide the tension responses. This avoids any effects of rate changes upon the tension responses (Koch-Weser & Blinks, 1963). A preliminary report of this work has been presented to the British Pharmacological Society (Broadley & Lumley, 1975).

## Methods

Guinea-pigs of either sex and weight range 400–600 g were killed by a blow on the head and exsanguinated under running water. The thorax was rapidly opened and the right and left atria removed separately. The right atrium was removed first, with cotton loops tied through the inferior vena cava and the atrioventricular junction which secured it to the perspex tissue holder (Blinks, 1966); a third cotton thread was passed through the superior vena cava for connection to an isometric transducer (Ether, Type UF 1, 28 g sensitivity range). To the left atrium was sewn an L-shaped stud which clamped it onto the tissue holder in close contact with a pair of punctate platinum electrodes. A cotton thread through the tip of the left atrial appendage passed to a second isometric transducer.

The atria, attached to the tissue holder, were immersed in an organ bath (50 ml) containing Krebs-bicarbonate solution having the following composition in g/l distilled water; NaCl 6.92, KCl 0.345, $CaCl_2 2H_2O$ 0.28, $NaHCO_3$ 2.1, $MgSO_4 7H_2O$ 0.29, glucose 2.0, $NaH_2PO_4 2H_2O$ 0.16. This was gassed with 5% $CO_2$ in $O_2$ and maintained at a temperature of $38 \pm 0.5$°C. Initial resting diastolic tensions of 1 and 0.5 g were applied to the left and right atria respectively. Records of force of contraction were obtained from the left atrium driven at a constant rate of 2.5 Hz with square wave pulses (5 ms and threshold voltage plus 50%) delivered by an SRI stimulator (Type 6053) and the tension developed was recorded on a Devices M19 polygraph. Rate of contraction was recorded from the spontaneously beating right atrium by means of a ratemeter (Devices, Type 2751) triggered by the tension signal.

After a 30 min stabilization period for the rate and tension to become constant, during which several changes of bathing medium were made, a preliminary cumulative dose-response curve to the agonist was obtained. The concentration was increased ten-fold until the maximum response was recorded. No difference in maximum response was found between cumulative and sequential administration of isoprenaline. After the tissue had been washed and the tension and rate had returned to their pre-drug levels, a second cumulative dose-response curve was constructed by increasing the concentration three-fold. Since it has been the experience of ourselves and others (Levy, personal communication) that the first dose-response curve to isoprenaline in guinea-pig atria is dissimilar in slope to subsequent curves, only the second, more detailed curve, was used for plotting purposes and calculation of the $EC_{50}$. The agonists under study were (–)-isoprenaline, histamine and calcium, but only with isoprenaline was a preliminary curve obtained. In some experiments with isoprenaline as the agonist, a third cumulative dose-response curve to salbutamol was obtained and comparisons were made between this and the second isoprenaline curve. The isoprenaline curves plotted in these experiments were corrected for any differences in sensitivity between the second (isoprenaline reference) and third (salbutamol) curve by performing control experiments in which three isoprenaline dose-response curves were obtained on the tissue. The mean difference in response size at each concentration was expressed as a percentage and this factor applied to the individual second isoprenaline curves. This predicted the sensitivity of the preparation to isoprenaline had the curve been obtained at the same point in time as the salbutamol curve.

Experiments were performed with atria from untreated animals and those pretreated with reserpine. The reserpine-induced supersensitivity was examined by using several pretreatment schedules; (a) 0.05 mg/kg (i.p.) at 24 h, (b) 0.5 mg/kg at 24 h, (c) 5 mg/kg at 24 h and (d) 5 mg/kg at 72 h, 3 mg/kg at 48 h and 3 mg/kg at 24 h before the animals were killed. A measure of the level of depletion of noradrenaline caused by these reserpine-treatment schemes was obtained by exposing the preparations to a single dose ($0.8 \times 10^{-4}$M) of the indirectly acting sympathomimetic amine $\beta$-phenylethylamine (Burn & Rand, 1958) before starting the experiment at the end of the stabilization period. The rate and tension responses were compared with cumulative dose-response curves for this amine produced in atria from untreated animals.

Drugs used were: histamine acid phosphate (Sigma), (–)-isoprenaline bitartrate dihydrate (Ward Blenkinsop Ltd), $\beta$-phenylethylamine as the base (Koch-Light Laboratories), reserpine (BDH Ltd) and salbutamol (Allen & Hanburys Ltd). All solutions were freshly prepared in 0.9% w/v NaCl solution (saline) with the exception of reserpine which was dissolved in 20% ascorbic acid and 1 N NaOH giving a solution with a pH of 5.0.

## Results

The rate responses of the right atria and the tension responses of the left atria were measured as the increase above the pre-drug level at each concentration. The mean rate and tension increases of at least six preparations were then determined. To facilitate comparison of rate and tension on the same graph, the mean response sizes were expressed as a percentage of the maximum response and plotted as such. Assessment of any supersensitivity was made by comparing the mean molar $EC_{50}$ values for rate and tension between atria from untreated guinea-pigs and those receiving the various pretreatments. Geometric means, calculated as the mean of the logarithm of the individual $EC_{50}$ values, were compared statistically by Student's *t*-test, the results of which are presented in Table 1 with the geometric means and their 95% confidence limits.

Isoprenaline in untreated atria was rate-selective in that the rate curve was to the left of that for tension. The mean curves of 39 experiments are plotted in each part of Figure 1 for comparison with the rate and tension curves obtained in atria from animals receiving the four pretreatment schedules. Schedule (a), the lowest dose of reserpine used, produced no change in the tension curve but depressed the rate responses (Figure 1a) as shown by a significant ($P<0.005$) displacement of the $EC_{50}$ to the right (Table 1). On increasing the reserpine dose to 0.5 mg/kg in schedule (b), supersensitivity of the tension responses to isoprenaline was revealed as a shift of the curve to the left (Figure 1b) with a significant ($P<0.001$) reduction of the $EC_{50}$ (Table 1). The rate responses were no longer depressed but there was no sign of any supersensitivity. Further increase of the 24 h dose to 5.0 mg/kg in schedule (c) produced supersensitivity of both rate and tension responses and although the rate curve was shifted only slightly to the left, the fall in $EC_{50}$ value was significant ($P<0.01$). The tension curve however was displaced no further than with schedule (b), having an identical $EC_{50}$ value. When the reserpine-treatment programme was extended to three days there was an additional shift of the tension dose-response curve giving an $EC_{50}$ value of 2.2 nM compared with 13.4 nM in untreated atria. The rate curve was similarly moved more to the left than with the 24 h treatment. Therefore, as the severity and duration of the reserpine pretreatment increased so the rate and tension responses were progressively potentiated; however, the tension curves were apparently displaced to a greater extent thus tending to close the rate-tension difference seen in untreated atria. The dose-ratios for rate and tension geometric mean $EC_{50}$ values were 5.1 and 3.4 in untreated and three day pretreated atria respectively.

Since atria from untreated animals were used as the reference for the reserpine-treatment programmes, a check was made that any changes in sensitivity were not merely due to the injection procedure and the vehicle, which proved to be slightly irritant. A group of animals were therefore injected for three days with the reserpine vehicle and the positions of the iso-

**Table 1** Mean $EC_{50}$ values for rate and tension responses to isoprenaline and histamine of atria from untreated and reserpine-pretreated guinea-pigs

| | *Isoprenaline (nM)* | | *Histamine (μM)* | |
|---|---|---|---|---|
| *Reserpine pretreatment* | *Rate* | *Tension* | *Rate* | *Tension* |
| Nil | 2.6<br>(2.2–3.2) | 13.4<br>(11.6–15.5) | 1.15<br>(0.72–1.81) | 1.24<br>(0.53–2.95) |
| 0.05 mg/kg i.p. at 24 h | $5.3^{\dagger}$<br>(3.5–8.0) | $11.1^{NS}$<br>(7.7–15.8) | — | — |
| 0.5 mg/kg i.p. at 24 h | $3.2^{NS}$<br>(1.2–8.2) | $5.0^{*}$<br>(3.2–7.9) | $0.97^{NS}$<br>(0.72–1.31) | $1.37^{NS}$<br>(0.88–2.13) |
| 5.0 mg/kg i.p. at 24 h | $1.5^{\ddagger}$<br>(1.2–1.9) | $5.0^{*}$<br>(3.4–7.4) | $1.32^{NS}$<br>(0.86–2.03) | $0.83^{NS}$<br>(0.64–1.08) |
| 5.0 mg/kg at 72 h and 3.0 mg/kg i.p. at 48 and 24 h | $0.64^{*}$<br>(0.41–0.99) | $2.2^{*}$<br>(1.4–3.4) | $0.86^{NS}$<br>(0.59–1.25) | $1.45^{NS}$<br>(1.05–2.02) |

Geometric means and their 95% confidence limits are shown.
Significance levels for differences in $EC_{50}$ values between untreated and reserpine-pretreated atria determined by Student's *t*-test are represented by; NS, not significant; * $P<0.001$; † $P<0.005$ and ‡ $P<0.01$.

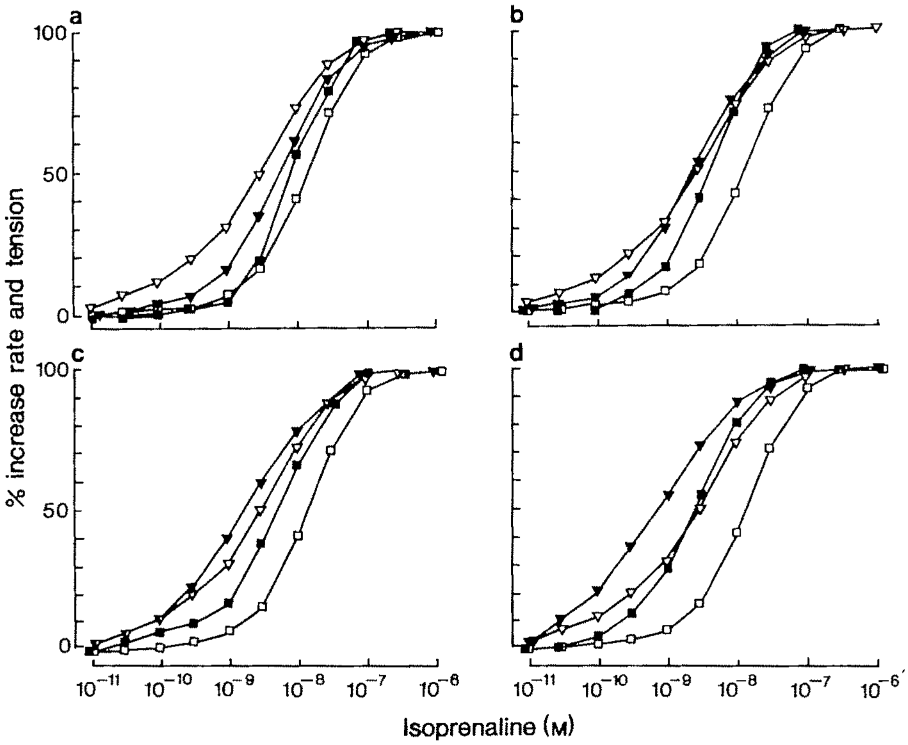

**Figure 1** Mean ($n=39$) cumulative dose-response curves plotted as a percentage of the maximum response for the increase in rate (▽) and increase in tension (□) responses to isoprenaline of isolated atria from untreated guinea-pigs. These are compared with the mean rate (▼) and tension (■) dose-response curves in atria from guinea-pigs pretreated with reserpine: (a) 0.05 mg/kg i.p. at 24 h ($n=10$), (b) 0.5 mg/kg i.p. at 24 h ($n=7$), (c) 5.0 mg/kg i.p. at 24 h ($n=10$), and (d) 5.0 mg/kg i.p. at 72 h and 3.0 mg/kg at 48 and 24 h before guinea-pigs were killed ($n=15$).

prenaline dose-response curves obtained in the atria from these animals ($EC_{50}$ values for rate 4.0 nM and tension 12.6 nM) were not significantly different from those from untreated animals ($EC_{50}$ values for rate 2.6 nM and tension 13.4 nM). The vehicle and injection procedure were therefore not responsible for the sensitivity changes encountered.

The supersensitivity has so far been measured with dose-response curves plotted as a percentage of the maximum response, where any changes in resting levels or absolute maximum reponses may be masked. However, a check on the reserpine pretreatments shown to induce supersensitivity to isoprenaline revealed resting rates of 245.7 ± 5.8, 245.4 ± 6.6 and 264.9 ± 5.3 beats/min after the 0.5 mg/kg, 5.0 mg/kg and three day pretreatments respectively compared with 235.5 ± 3.5 beats/min in untreated atria. The resting developed tensions after these pretreatments were 0.56 ± 0.06, 0.59 ± 0.06 and 0.78 ± 0.08 g respectively compared with 0.57 ± 0.03 g in the untreated atria. Therefore only after the three day pretreatment was there any significant difference ($P < 0.05$). The maximum absolute rate increases did not vary significantly between the three day and the 0.05, 0.5 and 5 mg/kg 24 h reserpine pretreatments (103.2 ± 5.5, 116.6 ± 7.2, 114.9 ± 7.4 and 119.6 ± 5.9 beats/min respectively). Similarly the absolute tension increases at the maximum did not differ significantly between the three day and 24 h pretreatments (0.69 ± 0.06, 0.66 ± 0.14; 0.72 ± 0.09 and 0.82 ± 0.13 g respectively).

Having satisfactorily demonstrated supersensitivity of both rate and tension responses to isoprenaline in our model, we continued with an examination of its selectivity by first obtaining dose-response curves to histamine in atria from untreated and reserpine pretreated animals receiving schedules (b), (c) and (d). Unlike isoprenaline, histamine was equiactive on rate and tension in untreated atria and these, almost superimposable dose-response curves, were compared with those from the pretreated atria (Figures 2a, b and c). The rate and tension dose-response curves remained virtually unaltered after each of the pretreatments. There was a slight shift to the left of the tension curve

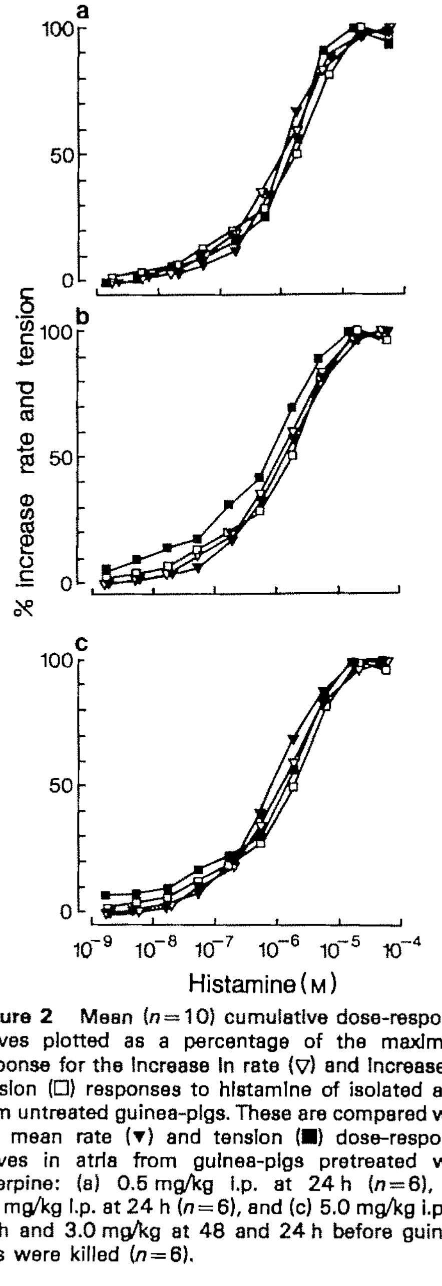

**Figure 2** Mean ($n=10$) cumulative dose-response curves plotted as a percentage of the maximum response for the increase in rate (▽) and increase in tension (☐) responses to histamine of isolated atria from untreated guinea-pigs. These are compared with the mean rate (▼) and tension (■) dose-response curves in atria from guinea-pigs pretreated with reserpine: (a) 0.5 mg/kg i.p. at 24 h ($n=6$), (b) 5.0 mg/kg i.p. at 24 h ($n=6$), and (c) 5.0 mg/kg i.p. at 72 h and 3.0 mg/kg at 48 and 24 h before guinea-pigs were killed ($n=6$).

after the 5 mg/kg dose of reserpine but the $EC_{50}$ value of this, and all the curves from pretreated atria, was not significantly different from the untreated atria values. Any small changes in position of the curves therefore could be attributed to scatter of the results.

The apparent selectivity of the supersensitivity for isoprenaline was further tested by examining $CaCl_2$, in this case only in untreated atria and those following the three day pretreatments schedule (d) (Figure 3).

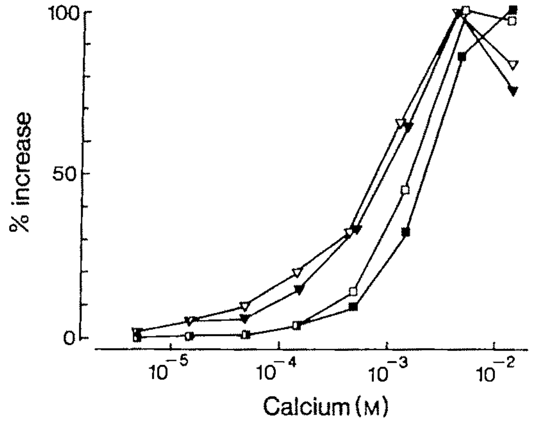

**Figure 3** Mean ($n=4$) cumulative dose-response curves plotted as a percentage of the maximum response for the increase in rate (▽) and increase in tension (☐) responses to $Ca^{2+}$ (calcium chloride) of isolated atria from untreated guinea-pigs. These are compared with the mean ($n=4$) rate (▼) and tension (■) dose-response curves in atria from guinea-pigs pretreated with reserpine 5.0 mg/kg i.p. at 72 h and 3.0 mg/kg at 48 and 24 h before they were killed.

This, the most effective pretreatment for inducing supersensitivity to isoprenaline, failed to alter the dose-response curves to calcium. If anything the tension curve was slightly depressed.

During the course of the study so far, each preparation had routinely received a test dose of the indirectly acting sympathomimetic amine β-phenylethylamine before starting the first dose-response curve. The rate and tension increase responses for each pretreatment were collected and the mean values compared with a full dose-response curve obtained in untreated atria (Figure 4). The selected dose produced approximately 50% of the maximum tension and almost a maximum rate response in untreated atria. After the three schedules (b), (c) and (d) the rate response was almost abolished, although the tension response was only halved. These responses were almost identical in the three groups of atria so that the three day pretreatment did not apparently have any further effect than the 24 h pretreatment. However, after the lowest dose of reserpine (0.05 mg/kg) the β-phenylethylamine responses were reduced to a lesser extent than by the higher doses.

Salbutamol is a partial agonist at the $\beta_1$-adrenoceptors of the heart (Brittain, Jack & Ritchie, 1970) and offered the opportunity to examine an alternative aspect of the reserpine-induced supersensitivity already demonstrated for isoprenaline; namely the effect upon the maximum response to this sympathomimetic amine. Salbutamol and isoprenaline dose-response curves were compared in the same preparations from untreated guinea-pigs or

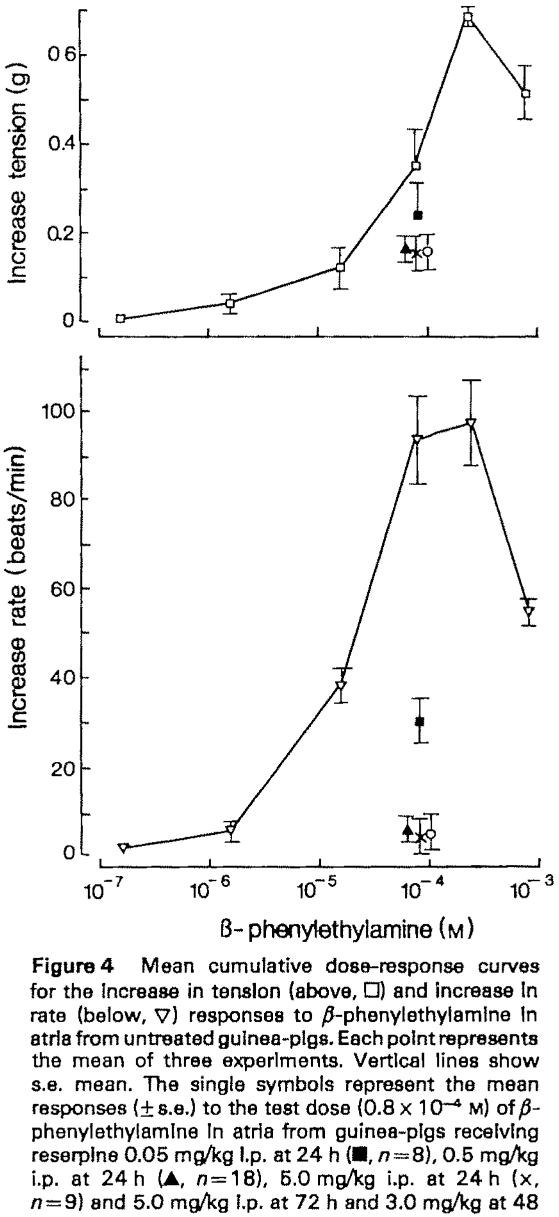

**Figure 4** Mean cumulative dose-response curves for the increase in tension (above, □) and increase in rate (below, ∇) responses to $\beta$-phenylethylamine in atria from untreated guinea-pigs. Each point represents the mean of three experiments. Vertical lines show s.e. mean. The single symbols represent the mean responses (±s.e.) to the test dose ($0.8 \times 10^{-4}$ M) of $\beta$-phenylethylamine in atria from guinea-pigs receiving reserpine 0.05 mg/kg i.p. at 24 h (■, $n=8$), 0.5 mg/kg i.p. at 24 h (▲, $n=18$), 5.0 mg/kg i.p. at 24 h (x, $n=9$) and 5.0 mg/kg i.p. at 72 h and 3.0 mg/kg at 48 and 24 h (O, $n=8$) before they were killed.

animals pretreated with 5 mg/kg reserpine (Figure 5) and the three day pretreatment (Figure 6), the salbutamol curves being plotted as a percentage of the isoprenaline maximum. In untreated atria, the maximum tension response to salbutamol was only 10.0±1.1% ($n=4$) that of isoprenaline, although it was more of a full agonist on rate at 63.3±3.4% of the isoprenaline maximum. However, in atria from animals receiving 5 mg/kg, the rate and tension maxima were raised to 72.6±5.1% ($n=4$) and 24.9±6.0% of the isoprenaline maxima respectively (Figure 5). After the three day pretreatment there was a further elevation of the maximum responses to 77.7±4.1% ($n=3$) and 44.7±5.7% ($n=4$) respectively (Figure 6). The salbutamol dose-response curves were also visibly shifted to the left by the reserpine pretreatment similarly to the isoprenaline curves. The three day pretreatment of Figure 6 in particular demonstrates the preferential reserpine-induced supersensitivity for tension responses, with the rate and tension dose-response curves to isoprenaline having moved closer.

## Discussion

The isolated left and right atria of the guinea-pig have been clearly shown to exhibit supersensitivity to isoprenaline after a range of reserpine pretreatments. This observation confirms previous reports of an enhanced sensitivity of both the positive inotropic (McNeill & Schulze, 1972) and chronotropic responses (Westfall & Fleming, 1968b; Taylor *et al.*, 1974) to catecholamines in isolated cardiac preparations. However, it does not conform to the opinion of Westfall & Fleming (1968b) that excessive trauma or dissection of the preparation obscures any supersensitivity, Neither they using a bath temperature of 38°C, nor Crout *et al.* (1962) using 32°C, could demonstrate sensitization of isolated atria. The dissection employed here was perhaps more extensive than that employed by these groups of workers. Although Westfall & Fleming (1968b) demonstrated some supersensitivity to the inotropic responses in perfused hearts, they have more recently proposed (Taylor *et al.*, 1974) that this is only apparent and due to concomitant rate increases that are known to exert positive inotropic effects themselves (Koch-Weser & Blinks, 1963). In perfused hearts electrically paced at a constant rate, they found no supersensitivity to the inotropic effects of noradrenaline. Our observations contradict this finding since they too were obtained in a paced preparation and unlike Taylor *et al.* (1974), the supersensitivity was apparent after only 24 h pretreatment and increased with extension of the pretreatment period. A possible explanation for the failure of Taylor *et al.* (1974) to find supersensitivity is the fact that they paced their hearts at 60 beats/min above resting rates. In our experience guinea-pig atrial preparations have resting rates of about 240 beats/min (238±3.1, $n=70$) at 38°C. At high frequencies the availability of immediate sources of energy may become a limiting factor in the amount of tension which may be developed so that drugs like acetylstrophanthidin produce little positive inotropic effect (Koch-Weser & Blinks, 1962). A frequency of about 5 Hz, as used by Taylor *et al.* (1974), could thus

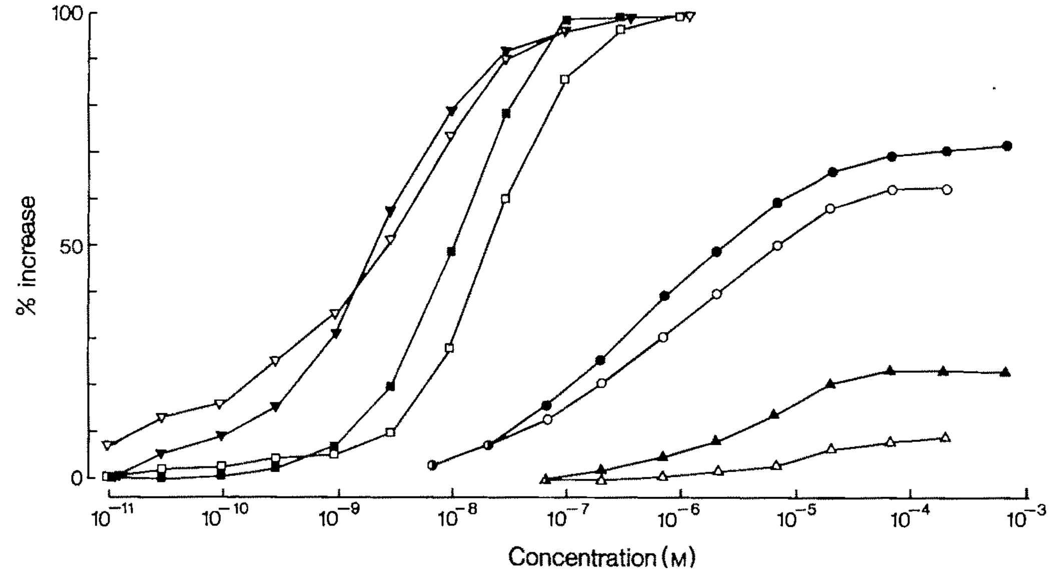

**Figure 5** Mean ($n=4$) cumulative dose-response curves to isoprenaline (increase rate, ▽; increase tension, □) and salbutamol (increase rate, ○; increase tension, △) plotted as a percentage of the isoprenaline maximum response in isolated atria from untreated guinea-pigs. These are compared with the mean ($n=4$) dose-response curves to isoprenaline (rate, ▼; tension, ■) and salbutamol (rate, ●; tension, ▲) obtained in atria from guinea-pigs pretreated with reserpine 5.0 mg/kg i.p. at 24 h before they were killed.

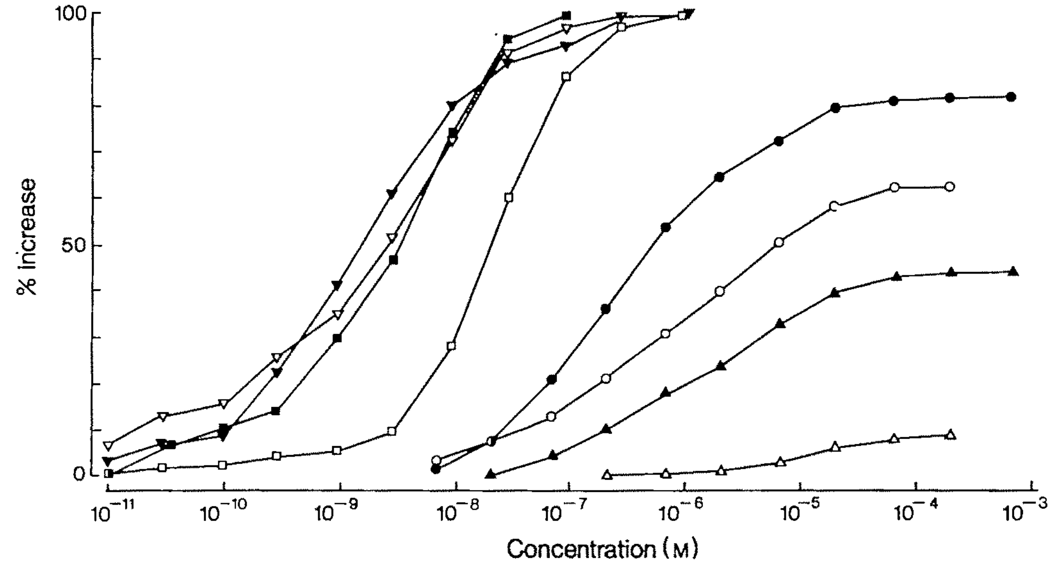

**Figure 6** Mean ($n=4$) cumulative dose-response curves to isoprenaline (increase rate, ▽; increase tension, □) and salbutamol (increase rate, ○; increase tension, △) plotted as a percentage of the isoprenaline maximum response in isolated atria from untreated guinea-pigs. These are compared with the mean ($n=3$) dose-response curves to isoprenaline (rate, ▼; tension, ■) and salbutamol (rate, ●; tension, ▲) obtained in atria from guinea-pigs pretreated with reserpine 5 mg/kg i.p. at 72 h and 3.0 mg/kg at 48 and 24 h before they were killed.

affect the inotropic action of the catecholamines and possibly mask any supersensitivity.

The sensitivity of the atria to the tension responses did not increase between the 0.5 and 5 mg/kg dose schedules, indicating perhaps that there is a maximum supersensitivity that may be achieved with 24 h pretreatment and that further increases in reserpine dose are without further effect. However, prolonging the pretreatment period did cause additional increase in sensitivity. This agrees with the findings of many other studies that the dosage and more particularly, the time of exposure to reserpine are important for supersensitivity (Fleming, McPhillips & Westfall, 1973). The importance of time of exposure has been stressed and most workers have found 24 h to be insufficient, although the cardiovascular system appears to become supersensitive more rapidly (Fleming & Trendelenburg, 1961), thus possibly explaining our results.

The positive chronotropic responses did not follow the same pattern. At the lowest dose of reserpine there was in fact depression of the rate responses to isoprenaline. Antagonism of sympathomimetic responses in rat atria following chronic administration of reserpine has been reported previously (Tiwari, Joshi, Inamdar & Sadre, 1966) and may be equivalent to our observation which would appear to be selective only for the pacemaker activity controlling rate. Only at the 5 mg/kg dose was there a significant potentiation of the rate responses and thereafter further potentiation occurred after the three day pretreatment. The supersensitivity was preferential for the tension responses at each of the reserpine-treatment schedules. This may be due to the depression which was seen only on the rate reponses with the 0.05 mg/kg dose counteracting any supersensitivity at the other pretreatment schedules. This subsensitivity may also increase with reserpine dose, thus preventing the supersensitivity for rate from achieving the same magnitude as that for tension. Alternatively the supersensitivity may be genuinely preferential for tension.

The question of the selectivity of the supersensitivity only for sympathomimetic amines now arises. The previously reported supersensitivity to the positive inotropic effects of noradrenaline in perfused hearts was accompanied by an increased sensitivity to histamine (McNeill & Schulze, 1972) and the positive chronotropic supersensitivity by an enhanced response to calcium (Westfall & Fleming, 1968b). On this evidence the supersensitivity of the heart was proposed to be non-specific. The present results are at variance with these views since neither the histamine nor calcium dose-response curves were displaced by the reserpine pretreatments. Our preliminary studies (Broadley & Lumley, 1975) described one instance of a shift of only the tension curve of histamine to the left following the 5 mg/kg 24 h pretreatment. However, further work with larger groups and with the three day pretreatment has shown that this result was erroneous and no supersensitivity to histamine could be detected with any of the pretreatments.

In tissues other than the heart there is more abundant evidence that the supersensitivity may be non-specific (Fleming *et al.*, 1973). However, there are also many reports of failure to induce supersensitivity to other agonists. For example the lack of supersensitivity to histamine in the aortic strip led Hudgins & Fleming (1966) to classify the supersensitivity as relatively non-specific.

In view of the divergence of our findings from previously held opinions, we were concerned that they could not be invalidated by the methods of expression of our results. As a quantitative measure of the degree of supersensitivity we have used the horizontal displacement of dose-response curves. Mean $EC_{50}$ values were compared statistically by the use of geometric means which are more valid than arithmetic means for examining sensitivity differences (Fleming, Westfall, De La Lande & Jellett, 1972). To obtain $EC_{50}$ values necessitated plotting the curves as a percentage of the maximum response. Kalsner (1974) has warned against the sole use of this method especially when the resting levels and absolute maxima may change after reserpine pretreatment. In the present work, the supersensitivity was observed in atria whose resting levels did not change. Similarly the absolute rate and tension increases at the maxima were of the same order after the various pretreatment schedules and therefore the progressive supersensitivity was not a function of the plotting method.

Finally, the possible mechanism of the sensitization of guinea-pig atria to isoprenaline, assuming it to be selective, must be considered. The supersensitivity has been causally related to the level of depletion of noradrenaline which thus interrupts the passage of nervous impulses to the organ, however depletion *per se* is not sufficient to induce supersensitivity since an additional time factor is also required (Fleming & Trendelenburg, 1961). In the present study a measure, albeit very limited, of neuronal depletion was obtained by recording the responses to the indirectly-acting sympathomimetic amine $\beta$-phenylethylamine in atria from each reserpine-treatment schedule. These were only partially prevented by the 0.05 mg/kg 24 h dose of reserpine which therefore could tentatively be concluded to have failed to deplete the noradrenaline satisfactorily; it also failed to induce supersensitivity. The subsequent dosage regimens all abolished rate responses indicating total depletion in the region of the SA-node, whereas the tension responses were only halved. By this test, depletion in the left atrium and myocardium could therefore be assumed to be incomplete and the 24 h pretreatment could not be bettered by three day exposure; it was presumably maximal. The noradrenaline stores of the left atrium therefore appear to be more resistant to depletion than those of the SA-node, which agrees with the findings of Blinks (1966). These results would suggest that the supersensitivity and depletion are not directly related since there was maximal depletion after the 0.5 mg 24 h dose, although the supersensitivity continued to develop with the dose (rate) and time of exposure (tension). It is also possible to obtain some depletion without supersensitivity. To achieve optimum depletion with minimal supersensitivity, the 24 h 0.5 mg/kg dose would appear to be suitable. To substantiate these claims, noradrenaline concentrations in the left and right atria would provide a better index of depletion.

Although in the past a prejunctional noradrenaline binding phenomenon was implicated in the supersensitivity (Kirkpekar, Cervoni & Furchgott, 1962), in recent years a postjunctional mechanism has been suggested with the non-selectivity lying at the cell membrane level (Trendelenburg, 1963; Fleming *et al.*, 1973). The present work has demonstrated sensitization to only sympathomimetic amines suggesting a more limited site of action than the common cellular pathways, possibly at the receptor itself. A change in the nature of the receptor can be discounted from a previous observation (Lumley & Broadley, 1975) that the $pA_2$ values of practolol are identical in atria from untreated guinea-pigs and those receiving the three day reserpine-treatment schedule used here. This agrees with findings of no change in affinity of phentolamine and phenylephrine for $\alpha$-adrenoceptors in aortic strips (Taylor & Green, 1971) and the nictitating membrane (Green & Fleming, 1967). The latter workers however found that the non-competitive antagonism of noradrenaline by phenoxybenzamine was reduced after 14 days of reserpine pretreatment. This was explained by either a change in the relationship between receptor occupation and response or by an increase in receptor numbers. A greater number of available receptors would permit noradrenaline to produce a maximum response in the presence of higher concentrations of phenoxybenzamine. The fact that $pA_2$ values remain constant does not however oppose the concept of receptor proliferation since they are derived from equal responses in the presence and absence of the antagonist involving the same number of receptors (Arunlakshana & Schild, 1959). They are therefore independent of the total receptor population (Green & Fleming, 1967).

The results obtained here with salbutamol may help to resolve this problem. The observation that the rate and tension responses to salbutamol fail to attain the same maximum as isoprenaline agrees with others (Brittain *et al.*, 1970; Hughson & Ledsome, 1975) and permits its classification as a partial agonist at the $\beta$-adrenoceptors of the heart (Stephenson, 1956). It can therefore be assumed to occupy all the available receptors when producing this reduced maximum response (Stephenson, 1956). Reserpine-treatment increased the maximum response compared with that of isoprenaline, but did not convert salbutamol to a full agonist. Presumably it therefore still occupied all the receptors, but the larger response might indicate that the number of receptors had increased. The rate response maximum to salbutamol was greater in each group of atria than the corresponding tension response maximum, which might also suggest that different numbers of receptors are involved in the right and left atria. This may be an alternative explanation for the preferential potentiation of the tension responses to isoprenaline by reserpine. If there were a greater receptor density in the right atrium than in the left atrium, which is possible in view of its denser innervation (Angelakos, Fuxe & Torchiana, 1963), then with each dose of isoprenaline a greater proportion might be occupied in the left atrium. Thus there are fewer spare receptors (Stephenson, 1956) and receptor proliferation might be expected to have a more marked effect upon the response size than in the right atrium where there may be more spare receptors and an increase in total receptor population would have proportionately less effect. This situation is analogous to non-competitive antagonism whereby the antagonist is irreversibly attached to a certain proportion of the receptors so effectively reducing the number of receptors, leading to a reduction of the maximum response. Thus changes in the total number of receptors can affect the size of the maximum response.

The raised maxima to salbutamol by reserpine may alternatively be explained not by receptor proliferation, but by an increased efficacy, whereby the same receptor occupation and affinity would generate a larger response. In fact Taylor & Green (1971) derived an equation that predicted an increased efficacy for phenylephrine in the aortic strips of reserpine-treated rabbits.

In conclusion, reserpine-induced supersensitivity has been demonstrated in the isolated atria of guinea-pigs to both the rate and tension responses. This was found to be selective for the $\beta$-adrenoceptor agonists isoprenaline and salbutamol. Whether this is due to an increased affinity or efficacy or receptor proliferation has yet to be resolved, however, the partial agonist salbutamol may offer an opportunity to examine these possibilities.

This work was supported jointly by the Science Research Council and Allen & Hanburys Ltd. under the CAPS scheme. We are grateful to Dr G.P. Levy of that company for his helpful discussions and for the gift of salbutamol. We also thank Ward Blenkinsop for the generous gift of isoprenaline.

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# MODIFICATION BY CAPSAICIN AND COMPOUND 48/80 OF DYE LEAKAGE INDUCED BY IRRITANTS IN THE RAT

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**1** Concentration-related dye leakage produced by intracutaneous injections of irritants was measured in rats by an Evans blue technique.

**2** In rats pretreated with a total dose of 50 mg capsaicin over 4 days, the response to capsaicin, formalin, HCl, KCl, prostaglandin $E_1$, bradykinin and bradykinin with prostaglandin $E_1$ ($10^{-6}$ M) were greatly reduced, the responses to histamine and 5-hydroxytryptamine were slightly reduced and those to adenosine 5′-triphosphate (ATP) and compound 48/80 were unaffected.

**3** Pretreatment with intracutaneous injections of compound 48/80 (0.5 μg, 24 and 48 h previously) recuded the responses to ATP, compound 48/80, HCl, KCl, prostaglandin $E_1$, and bradykinin but did not affect those to histamine, 5-hydroxytryptamine or bradykinin with prostaglandin $E_1$ ($10^{-6}$ M).

**4** Responses to capsaicin and formalin produced spotted blueing extending over a large area and were suppressed by compound 48/80 in the smaller pretreated area only. Capsaicin responses were reduced with larger doses of compound 48/80 (total dose 15 μg).

**5** It is concluded that the production of neurogenic oedema involves both sensory nerves and mast cells.

## Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent principle of chillies, has been shown to cause desensitization to chemically-induced pain, leaving sensitivity to mechanical stimuli unaffected (see Jancsó, 1960). Capsaicin pretreatment of animals also resulted in abolition of the inflammatory response to certain irritants and it was concluded that these irritants produced their inflammatory effects in untreated animals by stimulating 'pain receptors' to cause release of a neurohumour (Jancsó, Jancsó-Gábor & Szolcsányi, 1967, 1968). The object of the experiments on capsaicin-desensitized rats reported here was a more quantitative examination of the extent to which sensory nerve stimulation contributes to the oedema produced by a variety of irritants. Since Kiernan (1972a) has suggested that mast cells are involved in antidromic vasodilatation, another series of experiments was performed in which rats were pretreated with compound 48/80, to establish the role of mast cells as mediators of neurogenic oedema.

## Methods

Oedema produced in rats by the intracutaneous injections of irritants was visualized by leakage of Evans blue from the circulation. Male Wistar rats, 100 to 150 g, were lightly anaesthetized with ether and the tail warmed in water at 45–50°C. Intravenous injections of a 2% solution of Evans blue in 0.9% w/v NaCl solution (saline), 2.5 ml/kg, were made into a lateral tail vein. Intracutaneous injections (0.05 ml) of the irritants tested were made on the abdominal skin shaved with commercial animal clippers fitted with a 1/10 mm shaving head. The conscious animals were left for 20 min at 25°C before killing by stretching the neck. After killing, the abdominal skin was cut out and pinned peritoneal side uppermost, to enable measurement of areas of blueing. Irritant substances tested were histamine, 5-hydroxytryptamine (5-HT), bradykinin, prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$, acetylcholine, carbachol, HCl, KCl, adenosine 5′-triphosphate (ATP), capsaicin, compound 48/80, and a combination of bradykinin and prostaglandin $E_1$. Dilutions of irritants injected were made in Tyrode solution of the following composition (g/l): NaCl 8.0, KCl 0.2, $MgCl_2$ 0.1, $CaCl_2$ 0.2, $NaH_2PO_4$ 0.05, $NaHCO_3$ 1.0. HCl and KCl were diluted in water to 0.15M (iso-osmotic), and further dilutions were made in Tyrode solution. For each substance tested, four doses of serial 1 in 2 dilutions and a Tyrode solution control were given, in 5 separate regions of the abdominal skin. Five rats were used for each series with rotation of the doses to eliminate variation between sites.

To measure the intensity of the response, extraction of the dye was undertaken in a mixture of 7 ml of acetone and 3 ml of 0.5% sodium sulphate solution over 24 h according to the method of Harada, Takeuchi, Fukao & Katagiri (1971). After centrifugation the amount of dye was measured as absorbance at 620 nm using a Zeiss spectrophotometer. To allow for possible variations in background blueing of the rats, a reference blank for the readings was obtained from each rat by extraction as above of a similar sized piece of abdominal skin, taken from outside the blue areas.

### *Capsaicin pretreatment*

Rats were pretreated with a 1% 'solution' of capsaicin made up in ethanol and Tween 80 according to the method described by Jancsó *et al.* (1967). This method produced a very fine suspension rather than a solution. Rats under ether anaesthesia were given 2.5 mg of capsaicin on the first day, and two doses of 5 mg in the morning and afternoon of the second day. Rats which experienced respiratory difficulty were given a dose of isoprenaline as an aerosol. Rats that survived the first doses (about 70% of animals) were given, without anaesthesia, a further 7.5 mg and 10 mg on the third day, and two doses of 10 mg on the fourth day, a total dose of 50 mg. Rats were tested with irritants on the fifth day. All injections of capsaicin were given subcutaneously on the back. Two control groups were pretreated with a solution that contained only Tween 80, ethanol and saline.

### *Compound 48/80 pretreatment*

Rats under light ether anaesthesia were pretreated intracutaneously on the shaved abdominal skin with 0.05 ml of a 10 µg/ml solution of compound 48/80, 48 and 24 h before testing at the same sites with irritants (total dose of compound 48/80, 1 µg). One group was also pretreated with 100 µg/ml, 96, 72 and 48 h before testing with capsaicin (total dose of compound 48/80, 15 µg). One control group was pretreated intracutaneously with saline 48 and 24 h before testing with compound 48/80.

### *Analysis of results*

Concentration-response lines were plotted by eye as absorbance against concentration on a logarithmic scale, through the mean responses at each concentration. During the course of the experiments, rapid assessment of the effect of treatments was obtained from *t* tests between the means of normal and pretreated groups at each concentration level. To obtain information on the overall effect of treatment on the concentration-response lines, two-way analysis of variance was used. Since it was apparent from the log concentration-response lines that some responses were fitted better by a curve than a straight line, partitioning of the concentration variance by the method of orthogonal contrasts was used to test the significance of curvilinear regression (the quadratic and cubic components). In those analyses where the interaction variance from the two-way analysis was significant, it was partitioned in a similar manner to determine whether there was any significant effect of treatment on the linear, quadratic or cubic components of the concentration variance.

### *Drugs*

The following drugs were used: acetylcholine chloride (Sigma); adenosine 5′-triphosphate disodium salt (Sigma); bradykinin triacetate (Sigma); capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) (Sigma); carbachol (BDH); compound 48/80 (Wellcome); Evans blue (Difco); histamine diphosphate (Sigma); hydrochloric acid, Analar, (Ajax); 5-hydroxytryptamine creatinine sulphate (Sigma); isoprenaline hydrochloride aerosol (Isuprel, Winthrop); potassium chloride, Analar, (Ajax) and prostaglandins $E_1$, $E_2$, $F_{2\alpha}$ tromethamine salt (Upjohn).

## Results

Concentration-response lines for dye leakage could be obtained for all irritant substances tested except prostaglandins $E_2$ and $F_{2\alpha}$, acetylcholine and carbachol. Prostaglandin $E_2$ did not consistently produce significant dye leakage in the range $6 \times 10^{-8}$ M to $6 \times 10^{-5}$ M. Dye leakage could not be demonstrated at all with prostaglandin $F_{2\alpha}$ in the range of $6 \times 10^{-7}$ M to $6 \times 10^{-4}$ M. Therefore prostaglandins $E_2$ and $F_{2\alpha}$ were excluded from further study. Rats tested with acetylcholine $10^{-7}$ M to $10^{-1}$ M occasionally showed blueing at the lower concentrations but this was not a consistent finding. No obvious blueing was obtained with carbachol in the range $10^{-7}$ M to $10^{-3}$ M. Since the higher concentrations of acetylcholine and carbachol rapidly killed the rats, no further tests were made with these agents.

### *Control experiments*

Tween 20, 40, 60 and 80 have been shown to be histamine liberators in the dog (Krantz, Carr, Bird & Cook, 1948), and 35% ethanol caused total histamine release from rat isolated mast cells (Bray & van Arsdel, 1961). Since the capsaicin suspension was made in Tween 80 and ethanol (see Methods section), a control series of experiments was performed on rats pretreated with the same amount of saline, Tween 80 and ethanol as those that had undergone capsaicin pretreatment. No significant difference was obtained in the concentration-response lines to capsaicin and compound 48/80 in animals that had undergone such pretreatment. It was therefore assumed that any effects observed after capsaicin pretreatment were due to capsaicin and not to the suspending vehicle. Similarly, intracutaneous pretreatment with Tyrode solution in an identical manner to compound 48/80 pretreatment (see Methods section), did not significantly alter the response to intracutaneous testing with compound 48/80.

### *Visual appearance of responses*

The visual appearance of the dye leakage response was qualitatively similar for most irritants except capsaicin and formalin. The dye leakage response to these substances extended over a larger area than that to the other irritants tested, though not across the midline of the abdomen, and was characterized by spotted blueing surrounding the area of injection.

### *Concentration-response lines*

In both the capsaicin and compound 48/80 series of experiments the mean responses plotted against log concentration for most irritants were fitted adequately by a straight line, as shown by the lack of significant quadratic or cubic components of the concentration variances, and significant linear components: $P<0.001$ for all irritants except bradykinin ($0.1>P>0.001$ and $0.05>P>0.01$), HCl (non-significant regression in the capsaicin series and $P<0.001$ in the compound 48/80 series), KCl ($0.01>P>0.001$ and $P<0.001$) and prostaglandin $E_1$ ($0.05>P>0.01$ in the compound 48/80 series), each of which had shallow log concentration-response lines (Figures 1 to 4). The exception was compound 48/80 where the curvature of the line (Figure 4) was reflected in a significant quadratic component of the concentration variance ($P<0.001$ and $0.01>P>0.001$) as well as a significant linear component ($P<0.001$). Although the responses to ATP appeared to fit a curve (Figure 3), there was no significant quadratic component of the concentration variance in either series. However, it is likely that a quadratic component was

**Table 1** Degree of reduction of dye leakage produced by pretreatment with capsaicin and compound 48/80

| *Irritant* | *Concentration range (M)* | *Pretreatment* | *Degree of reduction§* | *Significance of components of interaction variance‡* | |
|---|---|---|---|---|---|
| | | | | *Linear* | *Quadratic* |
| 5-HT (Summer) | $6.25 \times 10^{-7}$ to | Capsaicin | † | | |
| (Winter) | $5 \times 10^{-6}$ | 48/80 | 0 | | |
| Histamine | $5 \times 10^{-5}$ to | Capsaicin | † | | |
| | $4 \times 10^{-4}$ | 48/80 | 0 | | |
| Bradykinin | $1.25 \times 10^{-6}$ to | Capsaicin | †† | | |
| | $1 \times 10^{-5}$ | 48/80 | †† | | |
| $PGE_1$ (1st Summer) | $7.25 \times 10^{-7}$ to | Capsaicin | ††† | *** | |
| (2nd Summer) | $6 \times 10^{-6}$ | 48/80 | ††† | *** | |
| Bradykinin with | $1.25 \times 10^{-6}$ to | Capsaicin | ††† | ** | |
| prostaglandin $E_1$ ($10^{-6}$ M) | $1 \times 10^{-5}$ | 48/80 | 0 | | |
| Compound 48/80 | — | Capsaicin | 0 | | |
| (0.25–2.0 μg/ml) | | 48/80 | †† | *** | * |
| ATP | $5 \times 10^{-4}$ to | Capsaicin | 0 | | |
| | $4 \times 10^{-3}$ | 48/80 | ††† | *** | * |
| HCl | $7.5 \times 10^{-4}$ to | Capsaicin | ††† | ** | |
| | $6 \times 10^{-3}$ | 48/80 | †† | | |
| KCl | $1.25 \times 10^{-2}$ to | Capsaicin | ††† | ** | |
| | $1 \times 10^{-1}$ | 48/80 | †† | | |
| Capsaicin | $5 \times 10^{-5}$ to | Capsaicin | ††† | ** | |
| | $4 \times 10^{-4}$ | 48/80 (1 μg) | 0 | | |
| | | 48/80 (15 μg) | †† | | |
| Formalin | $3.62 \times 10^{-2}$ to | Capsaicin | ††† | ** | |
| | $3.0 \times 10^{-1}$ | 48/80 | 0 | | |

§ Degree of reduction on arbitrary scale—0; none; †, slight; ††, marked; †††, very marked. (All reductions were significant at $P<0.001$.)

‡ The effect of pretreatments on the shape of the log concentration-response lines is shown in the significance of the linear and quadratic components of the partitioned interaction variance derived from two-way analysis of variance. *, $0.05>P>0.01$; **, $0.01>P>0.001$; ***, $P<0.001$.

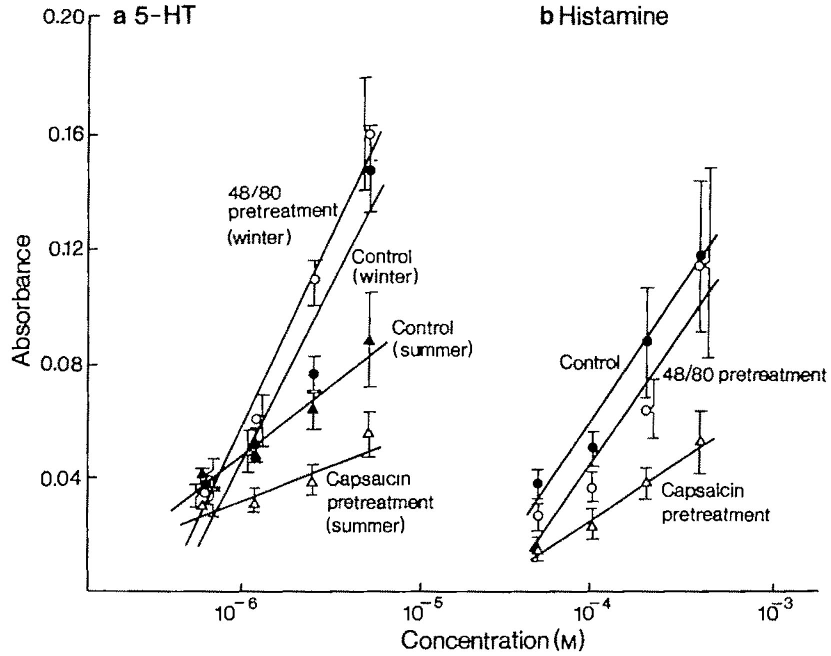

**Figure 1** Rat skin responses to concentrations (M) of (a) 5-hydroxytryptamine (5-HT) and (b) histamine measured as Evans blue leakage (absorbance). Effect of pretreatment with capsaicin and compound 48/80. Means ± s.e. of 5 responses. Mean control (winter controls in (a)) responses (●); capsaicin pretreated (△); compound 48/80 pretreated (○). In (a), summer control responses (▲) were used for comparison with the capsaicin pretreatment responses. Lines plotted by eye. Concentration on a logarithmic scale.

present in the response to ATP since compound 48/80 pretreatment produced a significant effect on the quadratic component of the interaction variance ($0.05 > P > 0.01$) (Table 1).

The effects of pretreatment with capsaicin and compound 48/80 on the dye leakage response produced by the irritants are summarized in Table 1 and are shown graphically in Figures 1 to 4. Although a set of control concentration-response lines was obtained for both the capsaicin and the compound 48/80 series of experiments, and these were used in the respective statistical analyses, only one control line is shown in most figures, since there was no significant difference between the two control lines. An exception was 5-HT where there appeared to be marked seasonal variation in the control response, the concentration used producing more marked dye leakage in winter than in summer (Figure 1). Marked seasonal variation also occurred with prostaglandin $E_1$. A concentration-related response was obtained from $7.25 \times 10^{-7}$ M to $6 \times 10^{-6}$ M in summer, but no response could be shown from $10^{-8}$ M to $10^{-3}$ M in winter. The results shown in Figure 2 were obtained in two successive summers. A seasonal variation in the constrictor response to prostaglandin $E_1$ was found previously (Chahl & Ladd, 1976a). The responses to bradykinin were markedly potentiated by the addition of prostaglandin $E_1$ ($1 \times 10^{-6}$ M) to the solutions ($P < 0.001$). Two control lines are shown for bradykinin in the presence of prostaglandin $E_1$, since the two series of experiments were performed at different times of the summer season (Figure 2).

### *Capsaicin pretreatment*

It can be seen that capsaicin pretreatment produced a partial reduction in the responses to 5-HT and histamine, a marked reduction in the responses to bradykinin and bradykinin with prostaglandin $E_1$, and almost complete abolition of the responses to prostaglandin $E_1$, HCl, KCl, capsaicin and formalin (Figures 1 to 4; Table 1). The abolition of the responses to the irritants was reflected in significant linear components of the interaction variances (see Table 1 for significance levels). The log concentration-response line to HCl from capsaicin-pretreated animals showed significant negative regression ($0.01 > P > 0.001$). These areas were blanched and this would explain the negative absorbance values compared with the blanks from skin outside the injected area. The responses to compound 48/80 and ATP were unaffected by capsaicin pretreatment (Table 1; Figures 3 and 4).

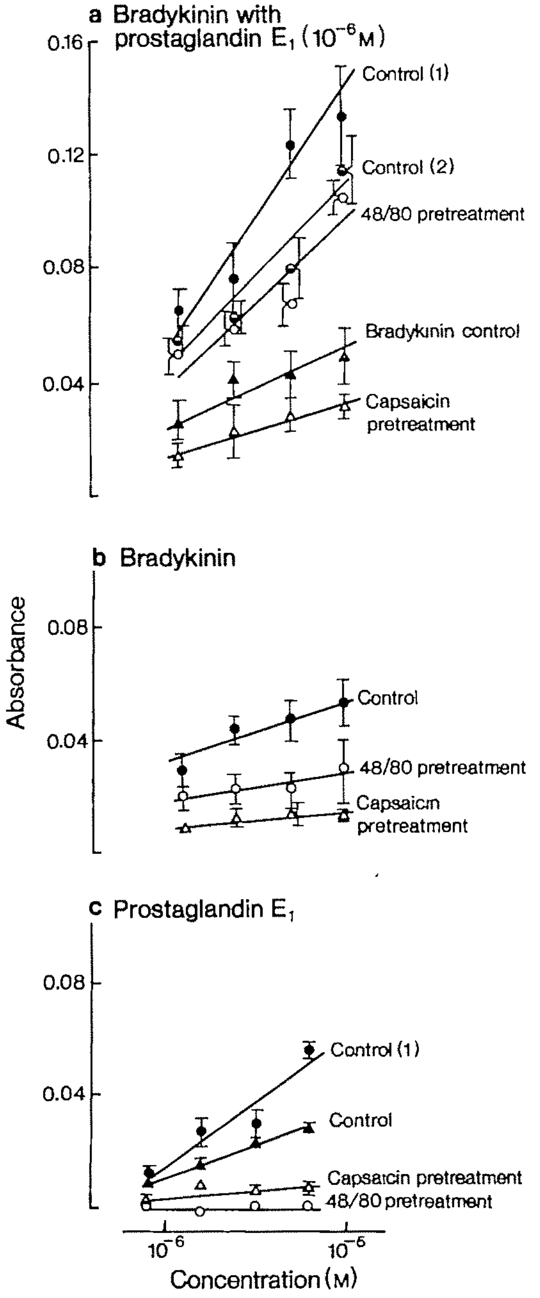

**Figure 2** Rat skin responses to concentrations (M) of (a) bradykinin with prostaglandin $E_1$ ($10^{-6}$ M) (b) bradykinin alone and (c) prostaglandin $E_1$ alone measured as Evans blue leakage (absorbance). Effect of pretreatment with capsaicin and compound 48/80. Means ± s.e. of 5 responses. Mean control (control (1) in (a) and (c)) responses (●); capsaicin pretreated (△); compound 48/80 pretreated (○). In (a), mean bradykinin responses (▲) were used for comparison with responses to bradykinin with prostaglandin $E_1$. Mean control (2) responses for bradykinin with prostaglandin $E_1$ (◓) were used for comparison with compound 48/80 pretreatment responses (see text). In (c), mean responses to prostaglandin $E_1$ (▲) were used for comparison with compound 48/80 pretreatment responses (see text). Lines plotted by eye. Concentration on a logarithmic scale.

### *Compound 48/80 pretreatment*

This pretreatment produced marked reduction in the responses to bradykinin, prostaglandin $E_1$, compound 48/80, ATP, HCl and KCl (Table 1, Figures 2, 3 and 4). Components of the interaction variance were significant for prostaglandin $E_1$, compound 48/80 and ATP (Table 1). It was observed that compound 48/80 pretreatment produced decreased blueing to capsaicin and formalin within the area of pretreatment, but outside this, the spotted blueing appeared more intense than that present in control animals. A group of rats pretreated with a higher dose of compound 48/80 (total dose, 15 μg) showed significant reduction in the total amount of dye leaked in response to capsaicin ($P < 0.001$) (Figure 4). The spotted blueing present in these pretreated animals extended over a similar area to that of the control animals but was much less intense.

## Discussion

Capsaicin pretreatment which has been shown to block neurogenic inflammation (Jancsó *et al.*, 1967) appeared to provide a useful tool for examining the neurogenic component of oedema produced by those substances which cause cutaneous pain and which therefore must stimulate some sensory nerves. In our experiments rats subjected to the capsaicin pretreatment described by Jancsó *et al.* (1967) still showed scratching and lachrymation when drops of capsaicin were placed on the cornea, and dye leakage when injected intracutaneously. Therefore the amount of capsaicin used and the duration of pretreatment was increased. These pretreated rats showed no response to application of capsaicin to the eye and did not respond with dye leakage when capsaicin ($5 \times 10^{-5}$ M to $4 \times 10^{-4}$ M) was given intracutaneously.

Both capsaicin and formalin when given intracutaneously produced a characteristic spotted blueing that did not extend across the midline of the abdomen. Other irritant substances tested produced a discrete circular area of blueing. The spotted blueing was similar in appearance to that observed by Chahl & Ladd (1976b) on antidromic stimulation of the saphenous nerve in rats. Therefore it would appear that spotted blueing is an indication of marked neurogenic oedema.

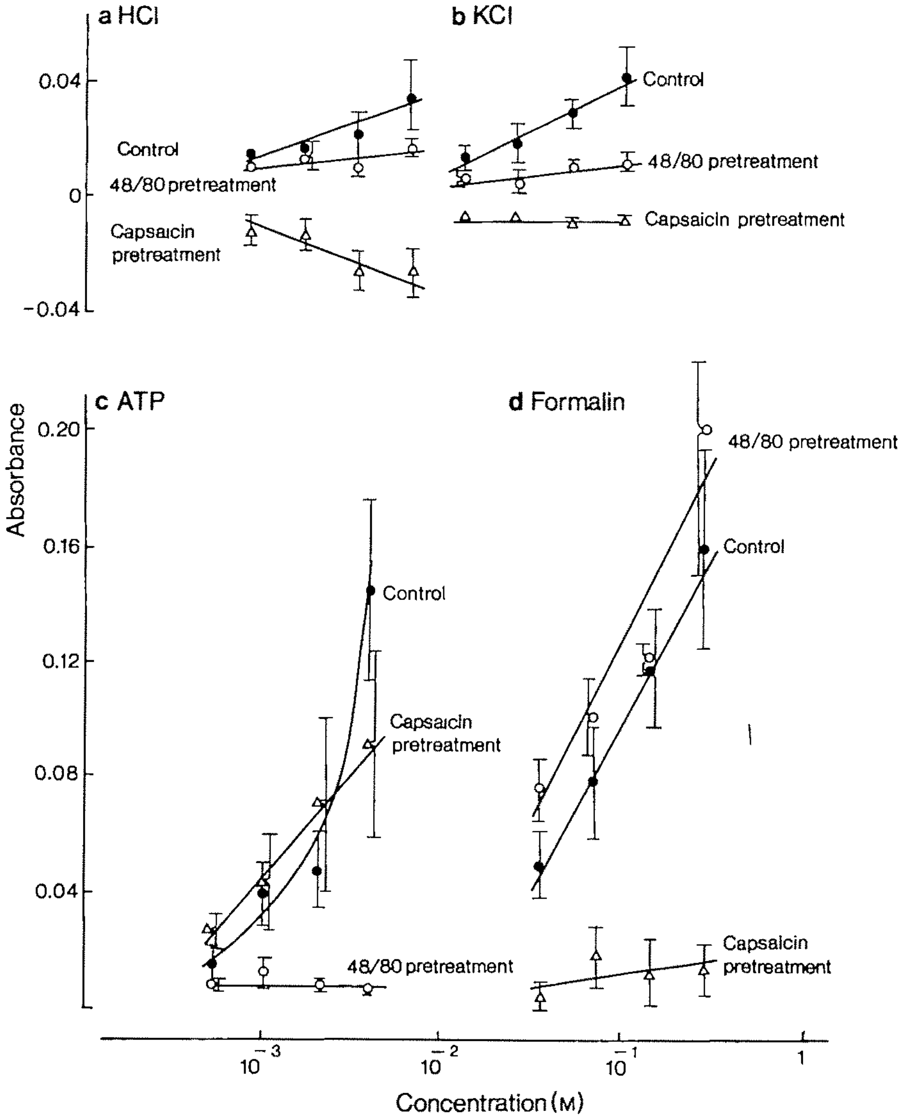

**Figure 3** Rat skin responses to concentrations (M) of (a) HCl, (b) KCl, (c) adenosine 5′-triphosphate (ATP) and (d) formalin measured as Evans blue leakage (absorbance). Effect of pretreatment with capsaicin and compound 48/80. Means ± s.e. of 5 responses. Mean control responses (●); capsaicin pretreated (△); compound 48/80 (○). Lines plotted by eye. Concentration on a logarithmic scale. Negative absorbance values occurred with skin blanching (see text).

Since the dye leakage responses to capsaicin, formalin, HCl, KCl and prostaglandin $E_1$ were abolished after capsaicin pretreatment, it is postulated that these substances, in the concentrations tested, exerted their inflammatory effects via sensory nerve terminals. A neurogenic component of the inflammatory response has been previously demonstrated for capsaicin (Jancsó *et al.*, 1967) and formalin (Brown, Kissel & Lish, 1968). Histamine, 5-HT, bradykinin and bradykinin with prostaglandin $E_1$ still produced some dye leakage after capsaicin pretreatment, indicating that these agents produce oedema through some other mechanism in addition to a neurogenic mechanism. Compound 48/80 and ATP were unaffected by capsaicin pretreatment, showing that dye leakage due to these substances was independent of neurogenic mechanisms.

Jancsó *et al.* (1968) postulated that a 'neuro-

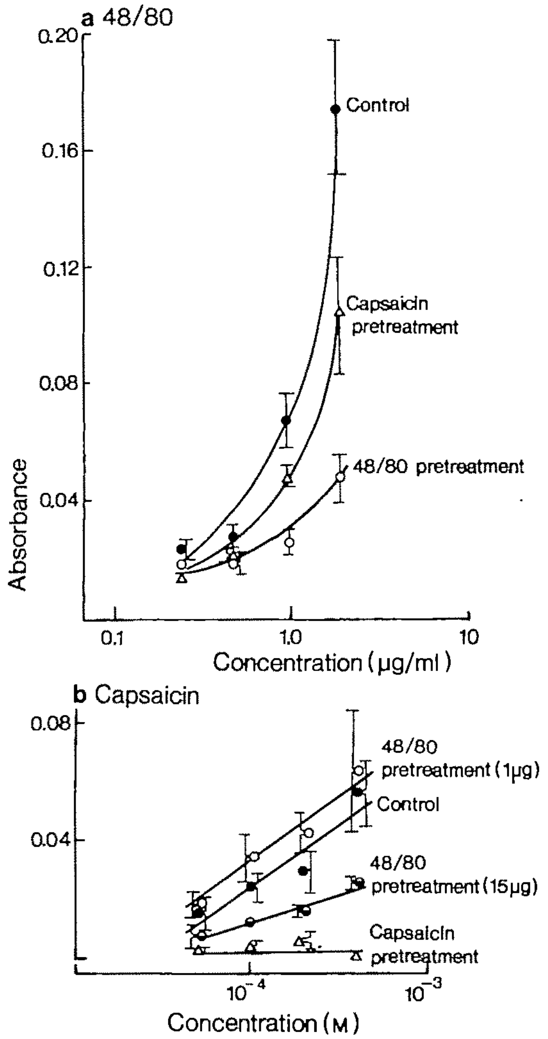

**Figure 4** Rat skin responses to concentrations of (a) compound 48/80 (μg/ml) and (b) capsaicin (M) measured as Evans blue leakage (absorbance). Effect of pretreatment with capsaicin and compound 48/80. Means ± s.e. of 5 responses. Mean control responses (●); capsaicin pretreated (△); compound 48/80 pretreated (○). In (b), mean responses (◉) were obtained on pretreatment with a higher dose of compound 48/80 (15 μg). Lines plotted by eye. Concentration on a logarithmic scale.

humour' is liberated on stimulation of sensory nerves. Kiernan (1972a) has demonstrated the degranulation of mast cells following antidromic stimulation of cutaneous nerves, and therefore it is possible that this neurohumour might exert its effects by causing degranulation of mast cells. After pretreatment with a dose of compound 48/80 sufficient to decrease the dye leakage response to compound 48/80 itself, it was found that the responses to ATP and prostaglandin $E_1$ were abolished, confirming previous findings that these substances exert their effects by causing release of amines from mast cells (ATP—Diamant & Krüger, 1967; Kiernan, 1972b; prostaglandin $E_1$—Crunkhorn & Willis, 1971). The responses to bradykinin, HCl and KCl were also reduced suggesting that part of their action is mediated through mast cells, either directly or via the release of some neurohumour from sensory nerve terminals.

If some part of the action of a substance liberated from sensory nerves is via mast cells, it would be expected that, if dye leakage was greatly reduced by capsaicin pretreatment, then it would also be reduced by compound 48/80 pretreatment. This was found to be so for bradykinin, prostaglandin $E_1$, HCl and KCl. The exceptions to this were bradykinin with prostaglandin $E_1$, capsaicin and formalin. Capsaicin and formalin showed reduced blueing within the smaller area of compound 48/80 pretreatment, but enhanced spotted blueing outside this area. Pretreatment with a higher dose of compound 48/80 (15 μg) produced an overall reduction in intensity, but not in the area of response to capsaicin. It is possible that the enhanced blueing was due to the low dose of compound 48/80 being sufficient to lower the threshold of amine release from mast cells in the skin surrounding the pretreated area, but insufficient to produce depletion of amines. It is also possible that compound 48/80 or substances released by it might have a potentiating effect upon the action of amines released by the neurohumour from the incompletely depleted mast cells. This potentiating effect might also explain the lack of reduction in response to bradykinin with prostaglandin $E_1$ by compound 48/80 pretreatment. Nevertheless it is difficult to understand why responses to bradykinin and prostaglandin $E_1$ were each reduced by compound 48/80 pretreatment but the prostaglandin-potentiated bradykinin response was not. Prostaglandin $E_1$ has been shown to potentiate histamine as well as bradykinin responses (Moncada, Ferreira & Vane, 1973; Williams & Morley, 1973) and it is possible that the combination of bradykinin and prostaglandin $E_1$ produced so much more stimulation of the sensory nerves than either bradykinin or prostaglandin $E_1$ alone, and also enhancement of the effects of the amines released from the partially depleted mast cells, that the effect of compound 48/80 was masked. It is concluded that those irritants which produce neurogenic oedema cause release of one or more substances from sensory nerve terminals, and that at least part of the oedema produced is mediated by amines released from mast cells (Figure 5).

The mechanism of capsaicin densitization remains unclear. It is most probable that C fibres mediate neurogenic oedema (Chahl & Ladd, 1976b) and it would seem reasonable to suggest that any substance which produces pain should produce some component

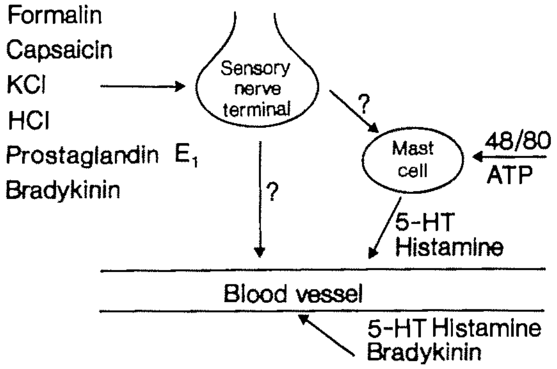

**Figure 5** Suggested sites of action of irritants.

of neurogenic oedema. However it is not known whether the mechanism of release of the neurohumour from sensory nerve terminals is linked to the mechanisms involved in the production of action potentials in the nerve. In these experiments the lack of consistent dye leakage response to acetylcholine was unexpected since it has been shown to produce pain on the cantharidine blister base (Keele & Armstrong, 1964). It has been suggested that fluoride-resistant acid phosphatase in the Rolando substance of the spinal cord is functionally related to the processing of nociceptive stimuli (Knyihár, László & Tornyos, 1974) and since the activity of this enzyme disappeared completely after capsaicin pretreatment in the rat (Jancsó & Knyihár, 1975) it has been suggested that the effect of capsaicin on pain receptors is exerted at the level of the first neurone in the nociceptive pathway. The present findings could equally well be explained by an action of capsaicin at the level of the sensory nerve terminal.

We wish to thank Dr J.E. Pike of the Upjohn Company, Kalamazoo, Michigan, U.S.A. for generous samples of prostaglandins and Miss Anne Schafferius for valuable technical assistance.

A preliminary communication of this work was presented to the 9th meeting of the Australasian Society of Clinical and Experimental Pharmacologists in November, 1975.

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*(Received April 20, 1976.
Revised June 21, 1976.)*

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# THE PHARMACOLOGICAL PROPERTIES OF THE CHOLINERGIC FALSE TRANSMITTER, *N*-2-ACETOXYETHYL-*N*-METHYLPYRROLIDINIUM, AND ITS PRECURSOR, *N*-2-HYDROXYETHYL-*N*-METHYLPYRROLIDINIUM

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1 The pharmacological properties of *N*-2-hydroxyethyl-*N*-methyl pyrrolidinium (pyrrolcholine) and its acetate ester, recently shown to be a false transmitter at the cholinergic electromotor synapses in *Torpedo marmorata*, also those of the corresponding morpholinium compounds (morpholinecholine, acetylmorpholinecholine), have been studied on the guinea-pig ileum, frog heart, frog rectus abdominis muscle, rat blood pressure, rat gastrocnemius muscle and dorsal muscle of the leech.

2 Acetylpyrrolcholine and acetylmorpholinecholine are full cholinoceptor agonists with dose-response curves parallel to that of acetylcholine. They are, however, less potent. Acetylpyrrolcholine is relatively more potent as a muscarinic drug (molar potency about 30% of that of acetylcholine in the ileum but only 4% on the leech) whereas acetylmorpholinecholine is more strongly nicotinic. The unacetylated compounds are very weak agonists with potencies comparable to that of choline.

3 Pyrrolcholine in high concentration showed a distinct neuromuscular blocking effect in the rat gastrocnemius muscle preparation. It is likely that this is a direct effect and not due to uptake by the presynaptic nerve terminals followed by conversion to a false transmitter since it was not reduced by hemicholinium-3, which is known to block uptake of choline and choline analogues by the presynaptic high affinity choline uptake system.

## Introduction

The concept of a 'false transmitter' is well established in the pharmacology of the sympathetic nervous system. Briefly, it is a substance which is taken up into adrenergic nerve terminals, and thence into synaptic vesicles, and on stimulation is released along with noradrenaline. Since many such substances are less active agonists than the natural transmitter, a partial blockade of synaptic transmission may result. The demonstration that only those substances which enter synaptic vesicles are released on stimulation has constituted important evidence for the 'vesicle hypothesis' in the adrenergic system (for review see Smith, 1972).

In principle, false transmitters should exist in the cholinergic system too, since it is unlikely that the storage mechanism in cholinergic terminals is completely specific for acetylcholine. However, the process by which acetylcholine is taken up into cholinergic synaptic vesicles is more complex and involves at least three steps: the entry of choline into the terminal via a high affinity choline permease (for references see Whittaker & Dowdall, 1975), the acetylation of choline to acetylcholine, a process known to be cytoplasmic in location (Fonnum, 1967) and the uptake of acetylcholine into the vesicles. The identification of false transmitters in the cholinergic system thus involves experiments with unesterified precursors, analogues of choline which are both taken up and acetylated.

Recent work has shown that the compound *N*-2-hydroxyethyl-*N*-methylpyrrolidinium (trivial name: pyrrolcholine) is a false transmitter precursor in the cholinergic electromotor system of *Torpedo* (Zimmermann & Dowdall, 1975a,b, 1976; Dowdall, Fox, Wächtler, Whittaker & Zimmermann, 1976) since the acetylated product is taken up into vesicles (as shown by subsequent isolation) and released on stimulation. Evidence for release from mammalian cholinergic endings is also forthcoming (Glick, Crane, Barker & Mittag, 1975; Collier, Barker & Mittag, 1976). A knowledge of the pharmacological properties of this compound and its acetate ester is thus of interest. Only a brief study of the acetate has so far appeared (Cho, Jenden & Lamb, 1972). In the study now to be described another putative false transmitter precursor *N*-2-hydroxyethyl-*N*-methyl morpholinium (trivial name: morpholinecholine) and its acetate ester have also been investigated for comparison.

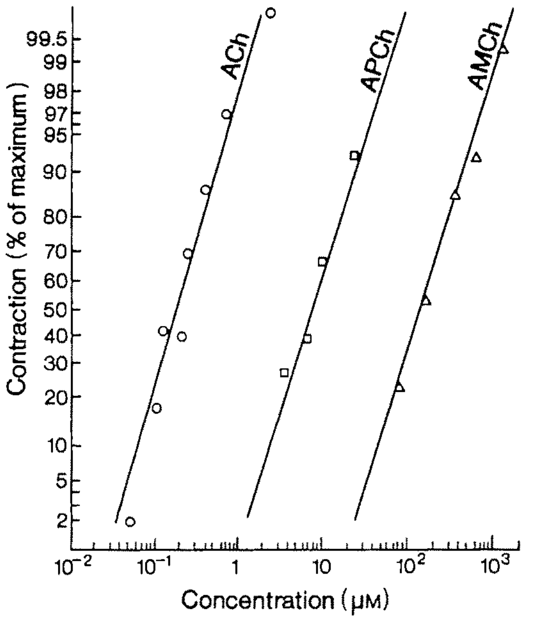

**Figure 1** Dose-response curves for (○) acetylcholine (ACh), (□) acetylpyrrolcholine (APCh) and (△) acetylmorpholinecholine (AMCh) applied to the dorsal muscle of the leech. Ordinates: percentage of maximum contraction plotted on a linear scale of probits; abscissae: logarithmic scale of doses.

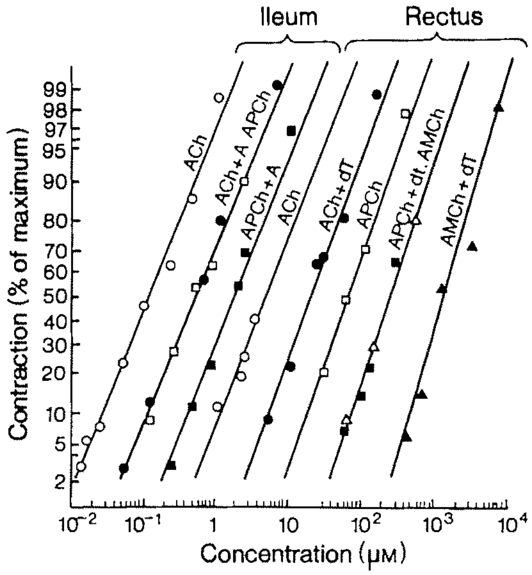

**Figure 2** Plots 1–3: effect of (filled symbols) atropine sulphate (A; 0.01 μM with respect to atropine) on the dose-response curves of (○) acetylcholine (ACh) and (□) acetylpyrrolcholine (APCh) applied to the guinea-pig ileum. Plots 4–8: effect of (filled symbols) (+)-tubocurarine chloride (dT; 0.1 μM with respect to base) on the dose-response curves of ACh, APCh and, in a different experiment, (△) acetylmorpholinecholine (AMCh) applied to the frog rectus abdominis muscle. Note that the blockade of the nicotinic effects of APCh and AMCh by dT and that of the muscarinic effect of APCh by A is about the same as that produced on ACh. Ordinates and abscissae as in Figure 1.

## Methods

### *Materials*

Pyrrolcholine and morpholinecholine were synthesized as iodides according to Barker & Mittag (1975); they were acetylated with acetylchloride according to Widlund & Heilbronn (1974) and the acetylation reaction was followed by the hydroxamate method (Hestrin, 1949). Acetylcholine perchlorate was used as a reference compound.

### *Pharmacological preparations*

The following preparations were used: leech dorsal muscle, frog rectus abdominis muscle, guinea-pig ileum (all as described by Whittaker & Barker, 1972), perfused frog heart, (Burn, 1952), rat blood pressure and sciatic nerve-gastrocnemius muscle preparation (Chiou, 1974).

For the last preparation Wistar rats (250–300 g) were anaesthetized with sodium pentobarbitone (35 mg/kg). The carotid artery was cannulated for blood pressure determination and the jugular vein for drug injection. The effect of the drugs on neuromuscular transmission was determined by recording isotonic contractions of the gastrocnemius muscle in response to supramaximal electrical stimulation once every 10 s through the sciatic nerve. Rectangular wave stimuli of 0.4 ms duration and 6–8 V strength were delivered by a stimulator through shielded platinum electrodes 4 mm apart placed on the nerve distally to a crushed region. The hypotensive responses of drugs were recorded simultaneously along with the neuromuscular effect. Dose-response curves were constructed for all substances and their $ED_{50}$ values were determined by probit plot analysis (Litchfield & Wilcoxon, 1949).

The $LD_{50}$ values of pyrrolcholine and acetylpyrrolcholine were determined in mice (20–25 g) according to Litchfield & Wilcoxon (1949). The drugs, dissolved in 0.9% w/v NaCl solution (saline), were given intraperitoneally in a volume of 0.1 ml.

## Results

The results obtained with the various preparations mentioned above are summarized in Table 1; in columns 5–6 they are expressed as equipotent molar ratios and the figures are therefore inversely proportional to molar activities. Acetylpyrrolcholine and acetylmorpholinecholine are both full cholinoceptor agonists which reach an intrinsic activity of 1 (100% effect). Their dose-response curves are parallel to that of acetylcholine, and are shifted to the right (Figure 1), from which one may conclude that their affinity for the postsynaptic receptor is lower than that of acetylcholine. As can be seen from Table 1, acetylpyrrolcholine is a rather more potent muscarinic stimulant whereas acetylmorpholinecholine has a stronger effect at the nicotinic receptor. By contrast, the unacetylated choline analogues were very weak agonists with potencies comparable to that of choline.

The nicotinic effect of acetylpyrrolcholine and acetylmorpholinecholine is blocked by (+)-tubocurarine to approximately the same extent as acetylcholine. In all cases the dose-response curves are parallel and shifted to the right (Figure 2). The muscarinic effect of acetylpyrrolcholine can be blocked competitively by atropine (Figure 2).

As seen in Figure 3a,b the fall of blood pressure (lower tracings) in the rat is very short-lasting. Doses of acetylpyrrolcholine or acetylmorpholinecholine which halve the blood pressure produce only a very feeble blockade of neuromuscular transmission (upper traces). Only after pretreatment with atropine (0.25 mg/kg) and physostigmine (0.5 mg/kg; Figure 3c,d) is there a more marked reduction of the muscle contractions. This suggests that both esters are hydrolyzed by cholinesterases at about the same rate as acetylcholine. On the other hand, the precursors pyrrolcholine and morpholinecholine in high concentrations show a distinct neuromuscular blocking effect which is present without any pretreatment (Figure 4a,b). Morpholinecholine has no influence on the blood pressure whereas pyrrolcholine has a strongly pressor effect. In view of the very rapid response after drug injection it is unlikely that the effects observed are indirect and due to a false transmitter action. More likely what we are seeing is a direct reaction on the postsynaptic receptor. To test this further we repeated the experiments after pretreatment of the rat with hemicholinium-3 (1 mg/kg) to block the presynaptic uptake of the false transmitter precursor. Figure 5 shows that there is no failure of the pyrrolcholine effect on neuromuscular transmission as would be expected in the case of an indirect mechanism.

Preliminary studies of the toxicity of acetylpyrrolcholine and its precursor pyrrolcholine showed an $LD_{50}$, by intraperitoneal injection (95% confidence limits in parentheses), for acetylpyrrolcholine iodide, 76 mg/kg (65–87 mg/kg) and for pyrrolcholine iodide, 225 mg/kg (210–241 mg/kg). After administration of acetylpyrrolcholine the animals exhibited evidence of cholinoceptor stimulation (lacrimation, salivation and reduction of motility). Tremor was present only in some cases and then only very weakly.

## Discussion

In Table 2 the affinity of the two precursors for the synaptosomal high-affinity choline permease (as measured by their ability to inhibit choline uptake at low choline concentration) and their rate of acetylation by choline acetyltransferase are given to show that both compounds interact with the permease and are acetylated: however morpholinecholine is a poor substrate for choline acetyltransferase and a

**Table 1** Median effective doses ($ED_{50}$) and equipotent molar ratios, relative to acetylcholine, of acetylpyrrolcholine (APCh) and acetyl morpholinecholine (AMCh)

|  | $ED_{50}$ (μM)* |  |  | Equipotent molar ratio (ACh = 1)* |  |
|---|---|---|---|---|---|
| *Preparation, effect* | *Acetylcholine* | *APCh* | *AMCh* | *APCh* | *AMCh* |
| Leech muscle, contracture | 0.054 (8)<br>(0.036–0.083) | 1.5 (5)<br>(0.86–2.6) | 29 (2)<br>(11–76) | 28<br>(14–55) | 760<br>(200–2900) |
| Frog rectus, contracture | 4.7 (3)<br>(2.2–9.7) | 70 (3)<br>(35–150) | 230 (3)<br>(120–300) | 15<br>(5–41) | 280<br>(118–652) |
| Guinea-pig ileum, contracture | 0.14 (8)<br>(0.08–0.23) | 0.48 (4)<br>(0.22–1.1) | 1600 (4)<br>(670–2800) | 3.6<br>(1.4–9.1) | 7000<br>(2300–21 000) |
| Frog heart, slowing and reduction of beat | 1.9 (4)<br>(0.5–6.6) | 5.4 (4)<br>(1.4–20) | 4600 (4)<br>(1000–20 000) | 2.9<br>(0.7–13) | 2400<br>(440–12 700) |
| Rat blood pressure, fall† | 3.1 (4)<br>(0.6–18.3) | 7 (4)<br>(1.5–13) | 910 (2)<br>(189–4390) | 2.9<br>(0.4–21) | 395<br>(51–3000) |

* Mean values: the figures in parentheses after the mean values are the no. of experiments, those below are the 95% confidence limits.

† Values are expressed as nmol/kg body weight.

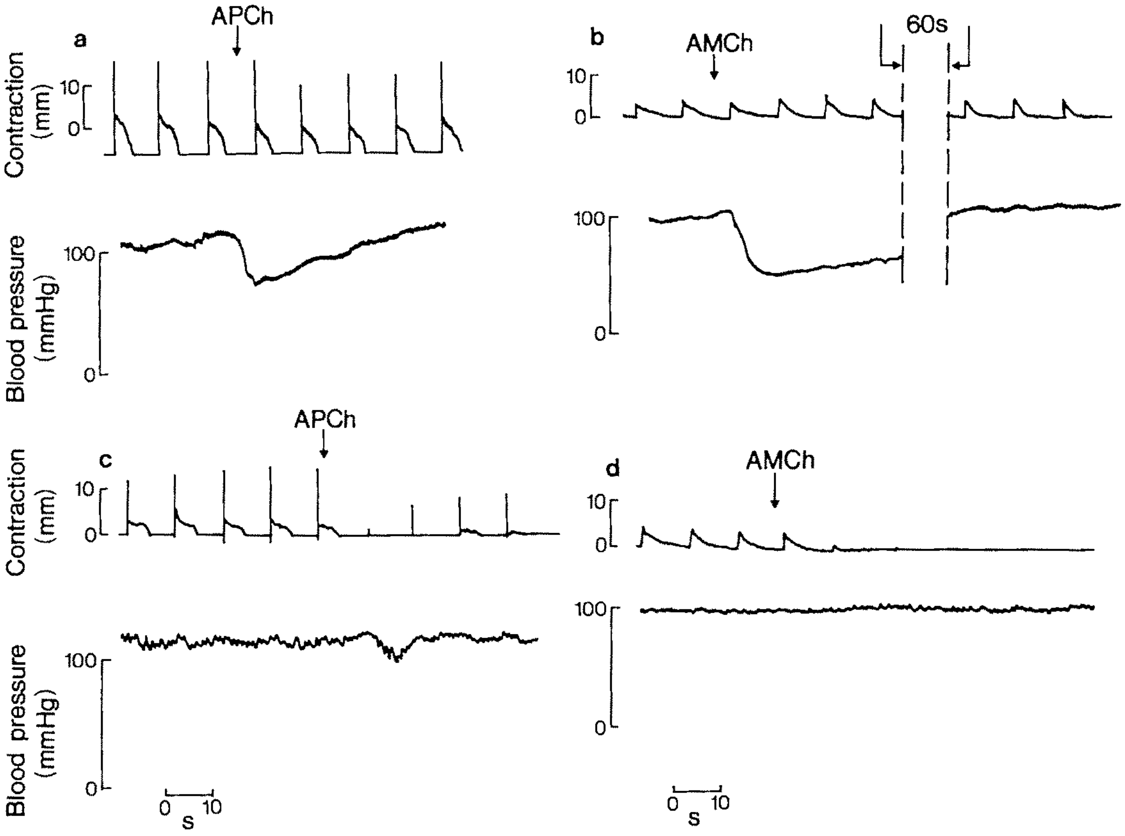

**Figure 3** (a) Effect of acetylpyrrolcholine (APCh; 15.5 µg/kg) on (upper trace contractions of the gastrocnemius muscle and (lower trace) blood pressure of the rat; (b) similar traces showing the effect of acetylmorpholinecholine (AMCh); (c, d) experiments similar to (a) and (b) except that the preparation was pretreated with atropine sulphate (0.25 mg/kg) and physostigmine sulphate (0.5 mg/kg).

**Table 2** The acetylation of *N*-hydroxyethyl-*N*-methyl-pyrrolidinium (PCh) and -morpholinium (MCh) methiodides by choline acetyltransferase and their inhibition of synaptosomal high-affinity choline permease

| *Source of preparation (Species, tissue)* | *Parameter (units)* | *PCh* | *MCh* | *Reference* |
|---|---|---|---|---|
| *Acetylation by choline acetyltransferase* | | | | |
| *Torpedo*, electric organ | $K_m$ (choline = 1) | 3.1 | 2.7 | M.J. Dowdall, unpublished |
| | $V_{max}$ (choline = 1) | 0.29 | 0.16 | |
| *Inhibition of synaptosomal permease* | | | | |
| Squid, optic lobe | $I_{50}$ (µM) at 1 µM choline | 6.8* | 100 | Barker, Dowdall & Mittag (1975) |
| Rat, striatum | | 3.7 | 37 | Simon, Mittag & Kuhar (1975) |
| *Torpedo*, electric organ | | 5.0 | 46 | M.J. Dowdall, unpublished |

* Calculated from $K_T$ and $V_{max}$ values for pyrrolcholine and choline

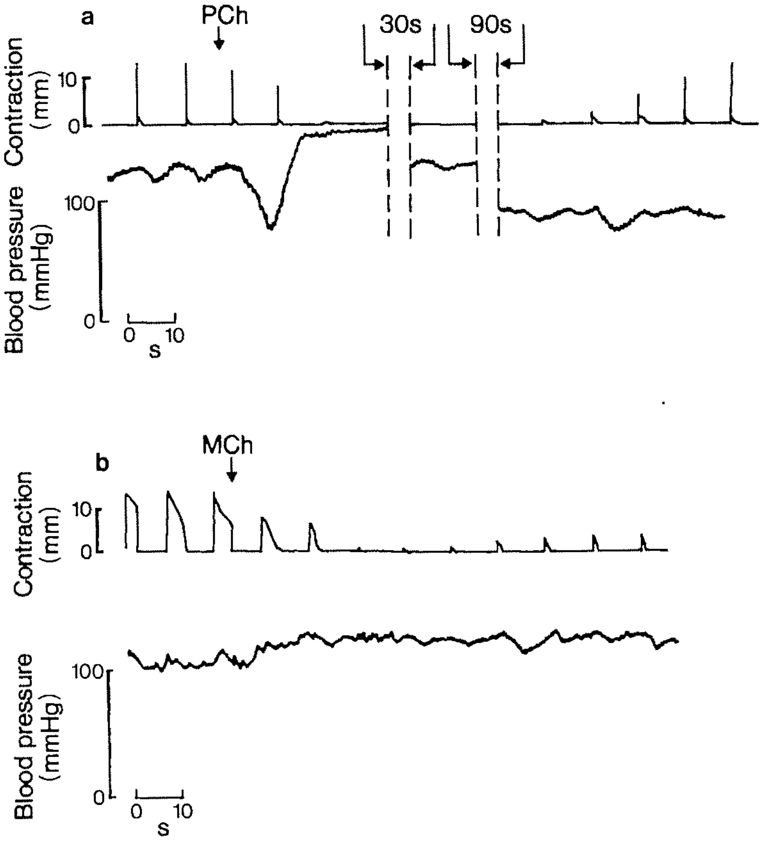

**Figure 4** Effect of (a) pyrrolcholine (PCh; 120 mg/kg) and (b) morpholinecholine (MCh; 128 mg/kg) on (upper traces) neuromuscular transmission and (lower traces) blood pressure in the rat. Note that in contrast to acetylpyrrolcholine, the effect of pyrrolcholine is to produce a sustained rise in blood pressure after an initial fall and both compounds show a more marked effect on neuromuscular transmission relative to their effect on blood pressure than (Figure 3) the acetylated compounds.

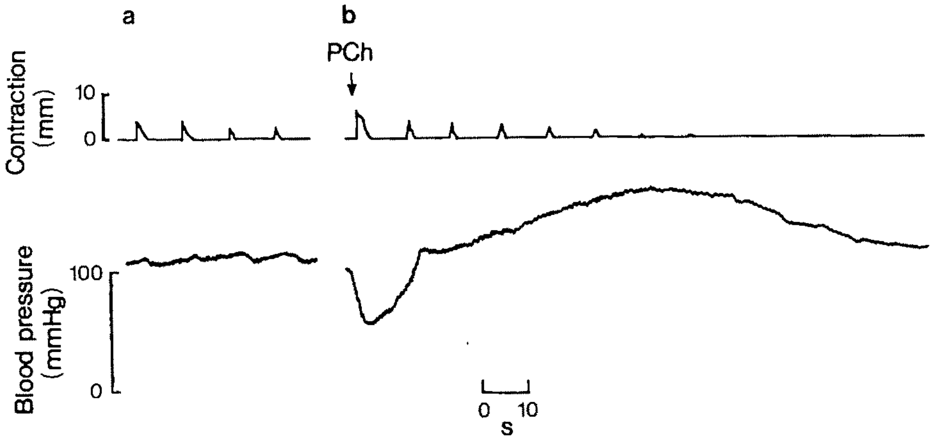

**Figure 5** (b) The effect of pyrrolcholine on (upper trace) neuromuscular transmission and (lower trace) blood pressure in the rat after injection of hemicholinium-3 bromide (1 mg/kg); (a) is a control trace before injection of pyrrolcholine. PCh = pyrrolcholine.

poor inhibitor of choline uptake; it is thus unlikely to compete effectively with choline for uptake and acetylation and its role as a false transmitter precursor has so far not been confirmed.

By contrast, pyrrolcholine is a somewhat better substrate for acetylation and is much more readily taken up into the terminals; in the *Torpedo* (Dowdall *et al.*, 1976) the acetylated product is known to be taken up into vesicles (by isolation of the vesicles and chromatographic identification of the ester) and to be released, along with acetylcholine, on stimulation, and there is evidence for release from mammalian cholinergic endings also (Glick *et al.*, 1975; Collier *et al.*, 1976). Since the acetylated product is considerably less potent as a cholinergic agonist than acetylcholine one might have expected pyrrolcholine to exert a synaptic blocking effect indirectly by way of conversion to a false transmitter. Although it did have a considerable blocking effect on the rat neuromuscular junction we were unable to demonstrate that this was other than a direct effect on the muscle cholinoceptors themselves. It remains to be seen whether effects consistent with a false transmitter precursor action would be uncovered at low doses and longer time intervals.

We are grateful to Dr. L.A. Barker for allowing us to refer to work in press and to Dr. M.J. Dowdall for providing compounds, for permitting us to include some of his unpublished results and for helpful discussions. The rat blood pressure and gastrocnemius experiments were done in the Pharmakologisches Institut of Göttingen University, by kind permission of Professor G. Schmidt.

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*(Received April 20, 1976.*
*Revised July 22, 1976)*

*Br. J. Pharmac.* (1977), **59**, 75–81

# THEOPHYLLINE AND PHENYLEPHRINE EFFECTS ON CARDIAC RELAXATION

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1 In the driven isolated left atrium of the rabbit theophylline shortened relaxation time in a similar manner to isoprenaline and histamine.

2 Phenylephrine lengthened relaxation time in a similar manner to calcium.

3 Theophylline caused phenylephrine to shorten relaxation time, which was inhibited by a $\beta$-adrenoceptor blocking drug, but theophylline did not potentiate the effect of phenylephrine on peak tension.

4 Theophylline separated drug effects on cardiac relaxation and contraction: in the presence of theophylline at a low calcium concentration, phenylephrine shortened relaxation time by $\beta$-adrenoceptor stimulation and increased peak tension by $\alpha$-adrenoceptor stimulation. At a high calcium concentration, theophylline potentiated the effect of isoprenaline, histamine and phenylephrine on relaxation time but inhibited the effect on peak tension.

## Introduction

The positive inotropic effect of agents such as catecholamines and histamine is accompanied by a rise in the myocardial concentration of cyclic adenosine 3′,5′-monophosphate (cyclic AMP), and it has been suggested that this nucleotide mediates the effect of the amines on contractility. An exact definition of the function of cyclic AMP may not be possible until the basic steps in contractile activation are completely understood. This study describes mechanical effects of two inotropic drugs in which an involvement of cyclic AMP remains controversial.

Catecholamines and histamine which stimulate the enzyme adenylate cyclase typically shorten relaxation time and abbreviate cardiac systole. Usually these agents increase relaxation velocity (or relaxation rate) more than agents which do not stimulate adenylate cyclase. The effect is presumably related to the stimulation by catecholamines of calcium sequestration in the sarcoplasmic reticulum (Katz, Taka & Kirchberger, 1975).

Theophylline and the related methylxanthine, caffeine, are believed to owe part or all of their effects to an inhibition of the enzyme cyclic nucleotide phosphodiesterase and the resulting increase in tissue cyclic AMP concentration (Sutherland & Rall, 1958; 1960). However, methylxanthines generally slow cardiac relaxation, which is compatible with the fact that these drugs inhibit calcium uptake by the sarcoplasmic reticulum (Nayler, Dunnett & Berry, 1975). Thus methylxanthines prolonged relaxation time in cat atrium (Blinks, Olson, Jewell & Braveny, 1972), cat papillary muscle (Blinks *et al.*, 1972; Henderson, Brutsaert, Forman & Sonnenblick, 1974), and rabbit interventricular septum (Shine & Langer, 1971). Also, caffeine inhibited the catecholamine effect on relaxation in rabbit papillary muscle (Gibbs, 1967) and cat papillary muscle (Blinks *et al.*, 1972). It has not been reported before that a methylxanthine can stimulate relaxation and potentiate effects of catecholamines and histamine on relaxation.

Methylxanthine effects on peak tension differ quantitatively in different species. Thus in cat papillary muscle, theophylline had no significant effect on peak tension below a concentration of 2 mmol/l, exerted its greatest effect on peak tension at 20 mmol/l, and prolonged systole in both of these concentrations (Blinks *et al.*, 1972). In guinea-pig atrium theophylline increased peak tension in concentrations as low as 0.17 and 1.7 mmol/l which did not prolong systole; 11 mM theophylline reduced peak tension and prolonged systole (Scholz & de Yazikof, 1971). Also, caffeine had a greater effect on peak tension in rabbit papillary muscle than in cat or dog papillary muscle (Bodem & Sonnenblick, 1975).

Agents which increase myocardial cyclic AMP concentration do not have a special effect on time to peak tension. Methylxanthine effects on time to peak tension differ qualitatively in different species. Methylxanthines shortened time to peak tension in rabbit interventricular septum (Shine & Langer, 1971) and rabbit papillary muscle (Bodem & Sonnenblick, 1975), did not change time to peak tension in guinea-pig atrium (Scholz & de Yazikof, 1971), and prolonged time to peak tension in cat atrium (Blinks *et al.*, 1972), cat papillary muscle (Skelton, Karch, Hougen, Marcus & Epstein, 1971; Marcus, Skelton, Grauer & Epstein, 1972; Blinks *et al.*, 1972; Henderson *et al.*, 1974; Bodem & Sonnenblick, 1975), dog papillary muscle (Bodem & Sonnenblick, 1975), and guinea-pig papillary muscle (Nayler *et al.*, 1975). It may be noted that caffeine shortened the action potential duration in rabbit atrium (Yanaga & Holland, 1969) but prolonged it in heart preparations of other species, e.g., the guinea-pig atrium (De Gubareff & Sleator, 1965).

Phenylephrine increases peak tension in isolated mammalian heart preparations without stimulating adenylate cyclase or raising the cyclic AMP concentration (Benfey, 1971; Osnes & Øye, 1975; Picken & Jarrott, 1975). In the driven left atrium of the rabbit phenylephrine increased peak tension and prolonged refractory period through α-adrenoceptor stimulation; in the spontaneously beating right atrium of the rabbit phenylephrine increased rate through β-adrenoceptor stimulation (Benfey, 1973). Effects of phenylephrine on the duration of cardiac systole have not been reported before.

## Methods

Strips of rabbit left atrium were suspended at 31 °C in a solution containing (mM): $Na^+$ 139.8, $K^+$ 5.9, $Mg^{2+}$ 1.2, $Ca^{2+}$ 0.9 or 3.2, $Cl^-$ 120.5 or 122.8, $HCO_3^-$ 24.9, $H_2PO_4^-$ 1.2, $SO_4^{2-}$ 1.2, glucose 10, and disodium edetate 0.03, which was aerated with 5% $CO_2$ in $O_2$. The muscles were stimulated with square-wave pulses of 1 ms duration and a voltage just above threshold at a rate of 1 Hz. Tension was recorded isometrically, and times to peak tension and to complete relaxation were estimated from oscilloscope recordings. Incubation with theophylline was for 20 minutes. Dose-response curves for isoprenaline, phenylephrine, and histamine were obtained cumulatively.

The drugs included theophylline, (–)-phenylephrine hydrochloride (K & K Laboratories), (–)-isoprenaline bitartrate dihydrate (Winthrop), histamine dihydrochloride (Hoffman-La Roche), and propranolol hydrochloride (Ayerst, McKenna & Harrison).

## Results

### *Theophylline*

In concentrations of 0.1, 0.3, and 1 mmol/l theophylline increased peak tension, contraction velocity (or rate of tension rise), and relaxation velocity, and decreased time to peak tension and time to complete relaxation (Figures 1 and 2). There were some exceptions: at the lowest concentration (0.1 mmol/l) theophylline did not shorten time to peak tension at 0.9 mmol/l $[Ca^{2+}]_0$, did not increase peak tension at 3.2 mmol/l $[Ca^{2+}]_0$, and did not increase contraction velocity or relaxation velocity; at the highest concentration (1 mmol/l) theophylline did not shorten time to peak tension.

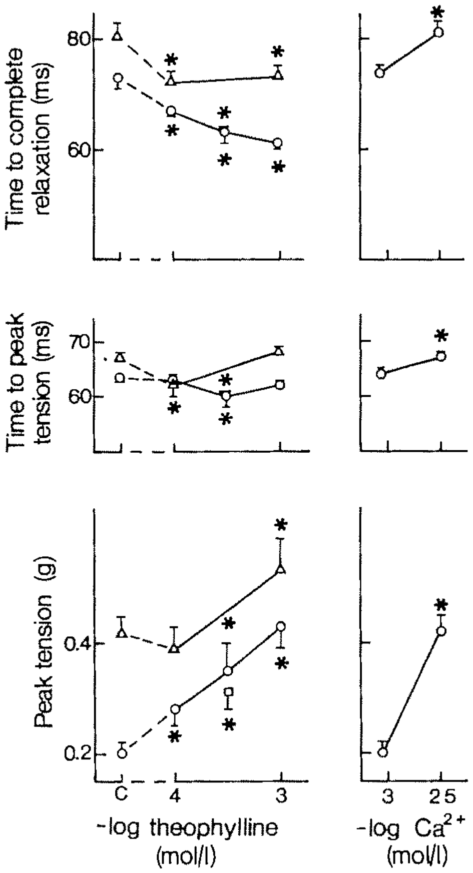

**Figure 1** Effects of theophylline at 0.9 mmol/l $[Ca^{2+}]_0$ in the absence (O) and presence of 1 μmol/l propranolol (□) and at 3.2 mmol/l $[Ca^{2+}]_0$ (Δ), and effects of calcium. Means of 9–24 experiments. Vertical lines show s.e. *$P<0.05$, compared to control (C or 0.9 mmol/l $[Ca^{2+}]_0$).

The effects of theophylline were not inhibited by the β-adrenoceptor blocking drug propranolol (Figures 1 and 2).

### *Calcium*

Increasing $[Ca^{2+}]_0$ from 0.9 to 3.2 mmol/l increased peak tension, time to peak tension, relaxation time, contraction velocity, and relaxation velocity (Figures 1 and 2).

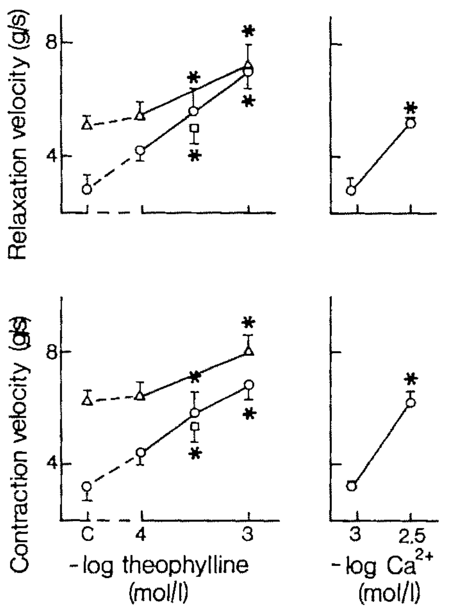

**Figure 2** As for Figure 1.

### *Isoprenaline*

Isoprenaline increased peak tension and reduced time to peak tension and relaxation time (Figure 3). All these effects were potentiated by theophylline at 0.9 mmol/l $[Ca^{2+}]_0$. Thus at 0.9 mmol/l $[Ca^{2+}]_0$ the $ED_{50}$ of isoprenaline for the effect on peak tension was 6.3 nmol/l in the absence, and 1.1 nmol/l in the presence, of 1 mmol/l theophylline.

At 3.2 mmol/l $[Ca^{2+}]_0$ the $ED_{50}$ of isoprenaline for the effect on peak tension was 2.2 nmol/litre. Theophylline potentiated the effects of isoprenaline on time to peak tension and relaxation time at the higher calcium concentration but inhibited the effect of isoprenaline on peak tension.

Effects of histamine were similar to those of isoprenaline, both in the absence and presence of theophylline, and at 0.9 and 3.2 mmol/l $[Ca^{2+}]_0$. The $ED_{50}$ of histamine for the effect on peak tension was 8.9 μmol/l in the absence, and 1.9 μmol/l in the presence, of 1 mmol/l theophylline at 0.9 mmol/l $[Ca^{2+}]_0$, and 5.6 μmol/l at 3.2 mmol/l $[Ca^{2+}]_0$.

### *Phenylephrine*

Phenylephrine increased peak tension and time to complete relaxation and did not significantly change time to peak tension (Figure 4).

Theophylline caused phenylephrine to shorten relaxation time, but theophylline did not potentiate the phenylephrine effect on peak tension. The $ED_{50}$ of phenylephrine for the effect on peak tension at 0.9 mmol/l $[Ca^{2+}]_0$ was 1.4 μmol/l in the absence, and 1.7 μmol/l in the presence, of 1 mmol/l theophylline. In the presence of theophylline and propranolol, phenylephrine did not shorten relaxation time. Propranolol did not inhibit the effect of phenylephrine on peak tension; the $ED_{50}$ of phenylephrine for the inotropic effect was 2.0 μmol/l in the presence of 0.3 mmol/l theophylline and 1 μmol/l propranolol.

At 3.2 mmol/l $[Ca^{2+}]_0$ the $ED_{50}$ of phenylephrine for the effect on peak tension was 0.93 μmol/litre. Theophylline caused phenylephrine to shorten time to peak tension and relaxation time at the high calcium concentration and inhibited the phenylephrine effect on peak tension.

## Discussion

It is not known why theophylline shortens relaxation time in rabbit atrium and lengthens relaxation time in rabbit ventricle. It may be assumed that theophylline stimulates calcium sequestration in rabbit atrium in a similar manner to isoprenaline and histamine. Whether this effect is due to inhibition of cyclic nucleotide phosphodiesterase and an increase in myocardial cyclic AMP concentration is not known. Theophylline has been reported both to increase cyclic AMP in guinea-pig heart (Kukovetz & Poch, 1971; Watanabe & Besch, 1974; Kukovetz, Poch & Wurm, 1975) and not to increase cyclic AMP in guinea-pig and rat heart (McNeill, Coutinho & Verma, 1974), rat heart slices (LaRaja & Reddy, 1969), and rat atrium (Birnbaum, Abel, Amidon & Buckner, 1975).

Theophylline potentiated the effect of noradrenaline (Rall & West, 1963) and histamine on peak tension (Kukovetz, Poch & Wurm, 1973), but theophylline did not potentiate the effect of phenylephrine on peak tension (Hamakawa, Shimizu & Toda, 1973). Thus there is a difference between agents that stimulate adenylate cyclase and phenylephrine which does not stimulate this enzyme. But there is no evidence that potentiation by methylxanthines is associated with an increase in cyclic AMP concentration. The potentiation of the inotropic effect of noradrenaline and histamine by theophylline was not accompanied by a rise in cyclic AMP (McNeill *et al.*, 1974).

It should be noted that inhibition of cyclic nucleotide phosphodiesterase(s) by methylxanthines interferes with the breakdown not only of cyclic AMP but also of cyclic guanosine 3′,5′-monophosphate (cyclic GMP) (Hardman & Sutherland, 1969; Goldberg, O'Dea & Haddox, 1973). Stimulation of guanylate cyclase by muscarinic agents increases tissue cyclic GMP levels (Goldberg, Haddox, Nicol, Glass, Sanford, Kuehl & Estensen, 1975). Acetylcholine increased the cyclic GMP concentration in guinea-pig ventricle, antagonized the inotropic effect

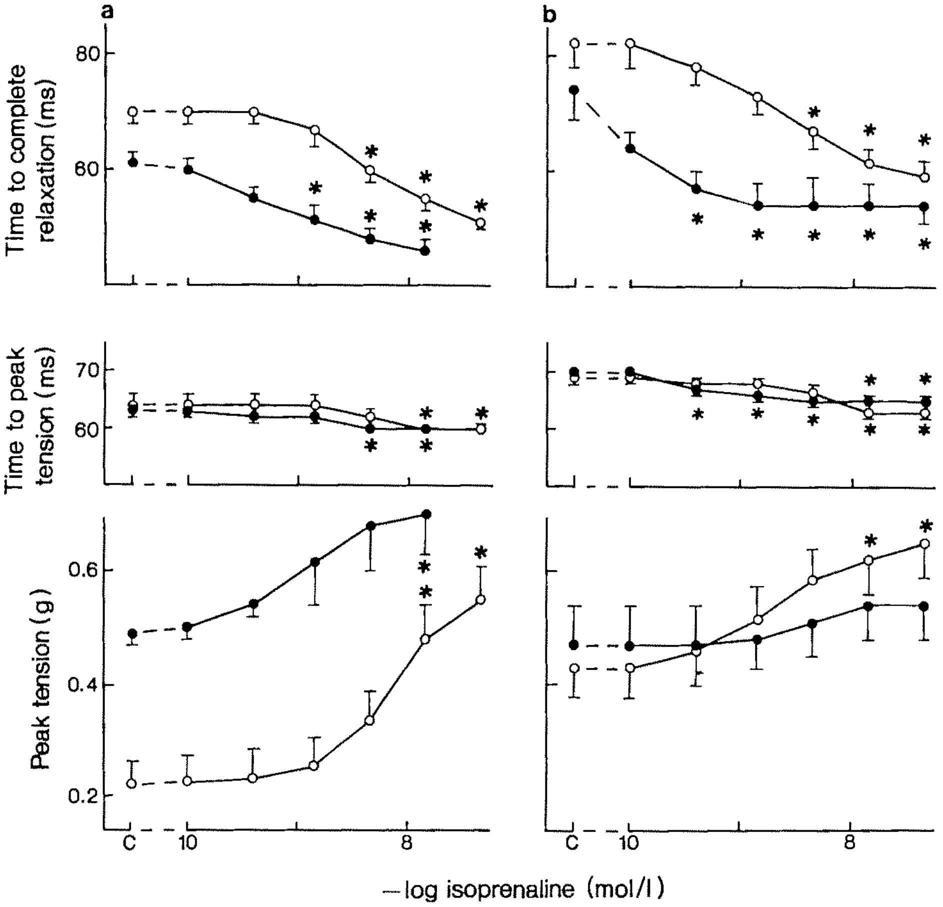

**Figure 3** Effects of isoprenaline (a) at 0.9 mmol/l $[Ca^{2+}]_0$ and (b) at 3.2 mmol/l $[Ca^{2+}]_0$ in the absence (○) and presence of 1 mmol/l theophylline (●). Means of 4–10 experiments. Vertical lines show s.e. $^*P < 0.05$, compared to control (C).

of isoprenaline and histamine, and did not inhibit the rise in cyclic AMP concentration produced by isoprenaline and histamine; it was suggested that cyclic GMP inhibits the inotropic effect of cyclic AMP (Watanabe & Besch, 1975). Can we assume that in the presence of theophylline the positive inotropic effect of cyclic AMP outweighs the negative inotropic effect of cyclic GMP? We have no data on myocardial cyclic GMP levels in the presence of theophylline.

There is controversy about the existence of calcium currents and the involvement of calcium-triggered calcium release from the sarcoplasmic reticulum in contractile activation. Catecholamines, histamine, and methylxanthines which increased cyclic AMP in guinea-pig hearts increased the rate of calcium entry via a slow inward current; ouabain or glucagon which did not increase cyclic AMP had no effect on the slow inward current (Thyrum, 1974; Watanabe & Besch, 1974).

It has been suggested that in cat, dog and rat heart contraction might be initiated largely by calcium released from stores near the myofilaments, most probably the sarcoplasmic reticulum, whereas in rabbit heart activating calcium might originate from superficial sites in the cell (Henderson *et al.*, 1974; Bodem & Sonnenblick, 1975). A methylxanthine might reduce the amount of calcium available to be released from internal stores by impairing sequestration, but might allow an increased calcium influx to exceed the concurrent sequestration rate during contraction so that the free calcium at the active sites rises. The effect of the methylxanthine on peak tension would then depend upon the relative contribution of calcium influx and calcium released from internal stores, and upon the extent to which these are affected by the methylxanthine.

Theophylline inhibited the effect of isoprenaline, histamine and phenylephrine on peak tension in rabbit

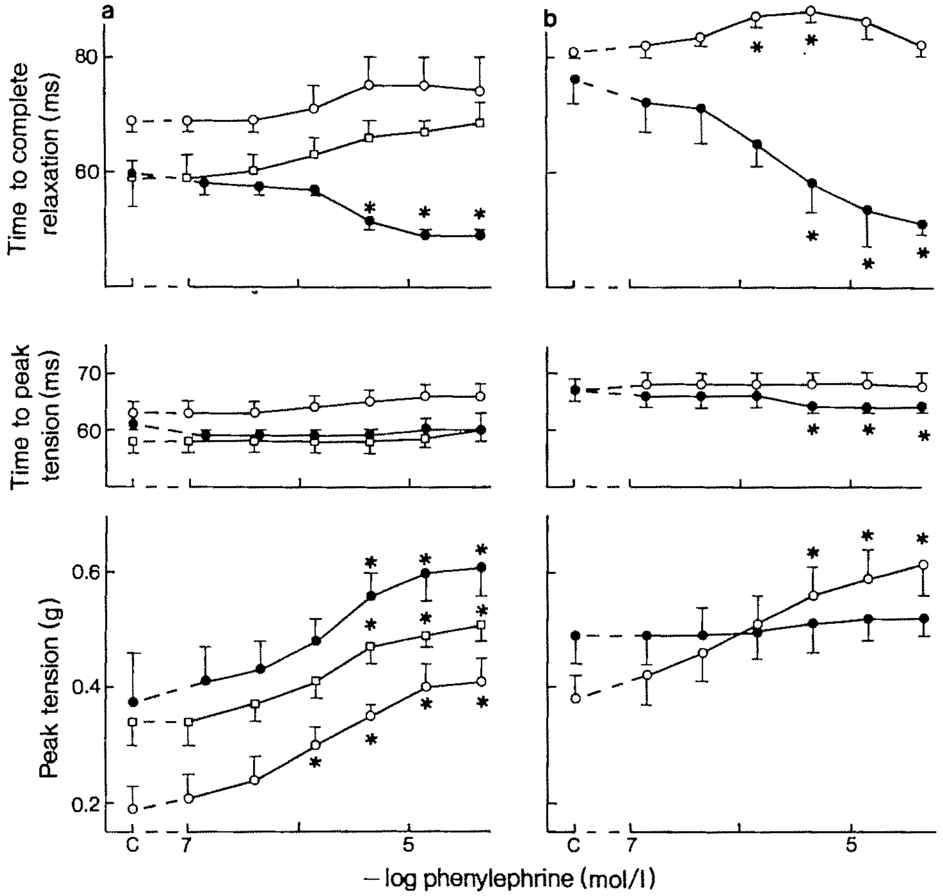

**Figure 4** Effects of phenylephrine (a) at 0.9 mmol/l $[Ca^{2+}]_0$ and (b) at 3.2 mmol/l $[Ca^{2+}]_0$ in the absence (○) and presence of 1 mmol/l theophylline (●) and 1 µmol/l propranolol and 0.3 mmol/l theophylline (□). Means of 4–9 experiments. Vertical lines show s.e. $^*P < 0.05$, compared to control (C).

atrium when the calcium concentration was high (3.2 mmol/litre). It could be suggested that the strong effect of the drugs on relaxation interfered with the effect on tension and terminated contraction prematurely (Reiter & Schober, 1965; Kavaler & Morad, 1966).

Contracture did not occur in rabbit atrium in the presence of theophylline at 3.2 mmol/l $[Ca^{2+}]_0$. In guinea-pig atrium 2.8 mmol/l theophylline, which produced the maximal increase in peak tension at 1.8 mmol/l $[Ca^{2+}]_0$, caused contracture when $[Ca^{2+}]_0$ was increased to 3.6 mmol/l (Scholz & de Yazikof, 1971).

In conclusion, it is evident that there are species differences in the mechanism of the inotropic effect of theophylline. Also, it appears that drugs exert their effect on relaxation independently of their effect on contraction, which has been proposed for catecholamines (Morad & Rolett, 1972).

This study was supported by a grant from the Medical Research Council of Canada.

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*(Received April 21, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 83–93

# THE SELECTIVE ACTION OF $\beta$-ADRENOCEPTOR BLOCKING DRUGS AND THE NATURE OF $\beta_1$ and $\beta_2$ ADRENOCEPTORS

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1 Purified membranes retaining a catecholamine responsive adenylate cyclase have been prepared from rabbit heart, lung and (pseudo-pregnant) uterus.

2 These preparations have the characteristics of plasma membranes and both heart and lung respond to $\beta$-adrenoceptor agonists in the order:

(±)-isoprenaline > (–)-noradrenaline > (–)-adrenaline > (+)-isoprenaline > salbutamol.

The sensitivity of the adenylate cyclase to $\beta$-adrenoceptor stimulation is improved by pre-treatment of the animals with reserpine and syrosingopine.

3 Dose-ratios for several concentrations of propranolol (non-selective $\beta$-adrenoceptor blocker), practolol and atenolol (cardio-selective $\beta$-adrenoceptor blockers) have been measured on all three membrane preparations. Schild plots of log (dose ratio $-1$) vs. log dose were virtually coincident for heart and lung with a dissociation constant ($K_b$) for propranolol very close to the pharmacological value. The ratio of $K_b$ values was 0.65 for practolol and 1.23 for atenolol compared with pharmacological cardio-selectivity ratios (measured on isolated atria and tracheal chain) of 67.6 and 110 respectively. The uterus/heart $K_b$ ratio was 51.5 for atenolol. Inhibition of the uterus by practolol gave a Schild plot with slope significantly less than 1, indicating a different mechanism of action from the heart.

4 $K_b$ values obtained by measuring adenylate cyclase stimulation in chopped tissue (including preparations of bronchial tree and alveolar tissue as well as whole lung) resembled the membrane values rather than those found in whole organs.

5 The results show that the pharmacological selectivity of practolol and atenolol is maintained at the receptor-adenylate cyclase level, at least as far as heart and uterus are concerned, though the smaller selectivity ratios in the biochemical system suggest that receptor difference is not the only factor and that phase distribution of the drug may also be important. Membranes prepared from whole lung show an overall $\beta_1$ response which may simply reflect the predominance of $\beta_1$ cell types containing $\beta_1$-adrenoceptors over bronchial smooth muscle.

## Introduction

The sub-division of $\beta$-adrenoceptor proposed by Lands, Arnold, McAuliff, Luduena & Brown (1967) was based on the varying responses of fat tissue, heart, lung and blood vessels to a number of agonists. The lipolytic response correlated well with cardiac stimulation and was classed as $\beta_1$, whereas bronchodilatation and vasodepression correlated with neither of these and were classed together as $\beta_2$. The implication was that these represented distinct receptor sub types and support for this concept was increased by the discovery of practolol (Dunlop & Shanks, 1968), a $\beta$-adrenoceptor blocking drug which has selective actions in the heart and has proved useful in the clinic for its ability to control cardiac dysrhythmias in asthmatic patients without producing bronchoconstriction (Van Durme, Dossaert, Vermeire & Pannier, 1973). A number of other considerations may affect selectivity, however, besides a true difference in receptor. Metabolism and lipid solubility may affect the gross distribution of the active species and diffusion barriers at the micro-environmental level may act to produce different concentrations of drug around identical receptors. Some attempts have already been made to characterize the $\beta$-adrenoceptors in biochemical terms and it now appears to be well established that responses to $\beta$-adrenoceptor stimulation are mediated in some way through the cyclic AMP system: $\beta$-adrenoceptor agonists are known to stimulate adenylate cyclase in cell-free preparations and their action can be blocked

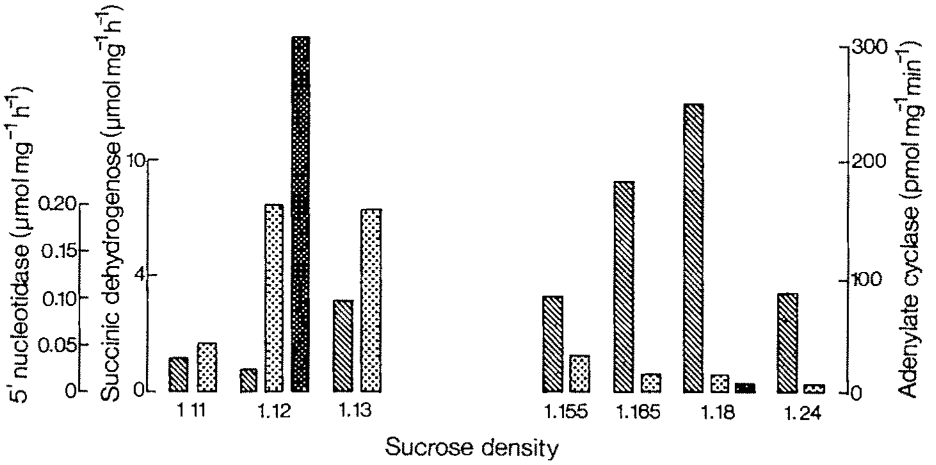

**Figure 1** Enzyme activity of sucrose density gradient fractions from rabbit ventricle. Diagonally-hatched columns=succinic dehydrogenase, dotted columns=5′nucleotidase, cross-hatched columns=adenylate cyclase.

competitively by $\beta$-adrenoceptor antagonists. This subject is reviewed by Robison, Butcher & Sutherland (1971). The cyclic AMP response has been used by Burges & Blackburn (1972) to examine the actions of selective agonists and blockers on homogenates of heart and lung and by Mayer (1972) for a similar study on heart and liver. Both these authors concluded that selective agonist and antagonist actions were still apparent at the tissue homogenate level. The purpose of the present study was to prepare more highly purified and characterized membrane fractions with $\beta$-adrenoceptor properties from $\beta_1$ and $\beta_2$ tissues and to make a quantitative study of their response to selective and non-selective $\beta$-adrenoceptor blocking drugs.

## Methods

### *Plasma membrane preparation: continuous density gradients*

New Zealand white rabbits (2–2.5 kg) were dosed 22 h before death with syrosingopine (4 mg/kg s.c.) followed 16 h before death with reserpine (4.5 mg/kg s.c.). For uterus preparations, females had previously been ovariectomized bilaterally under halothane anaesthesia and allowed to recover for one week. They were then dosed with oestradiol benzoate (4 µg/kg s.c. in arachis oil) for five consecutive days followed by 5 daily doses of oestradiol benzoate (0.4 µg/kg s.c.) and progesterone (2 mg/kg s.c. suspended in arachis oil).

After killing the animals by a sharp blow at the back of the skull, the tissues required were quickly excised. The ventricle was dissected free of excess fat, large vessels and atria and the lung freed from the trachea. The uterus was cut open longitudinally and the internal mucosal layer scraped off with a glass microscope slide. The tissues were then placed in cold sucrose buffer (mM): tris pH 7.4(5), $MgSO_4$(0.5), EGTA(1) in sucrose solution sp.gr. 1.04. All sucrose solutions are referred to by specific gravity at 20°C: refractive index was measured with an Abbé refractometer and the corresponding specific gravity obtained from tables. All subsequent stages of the preparation were at 4°C.

Tissue (4 g) was chopped with scissors into 150 ml of the sucrose buffer (sp.gr. 1.04) and homogenized with a Braun multimix blender for 4 periods of 5 s each at speed 1, then given 5 strokes with an air driven teflon glass homogenizer. The slurry was centrifuged for 10 min at 400 *g* in an MSE 18 centrifuge. The supernatant was put through 3 layers of cheesecloth and centrifuged at 177,000 *g* for 40 minutes.

The pellet was resuspended in 10 ml (ventricle or uterus) or 20 ml (lung) of sucrose buffer (sp.gr. 1.04). This homogenate (3 ml) was layered on to continuous sucrose density gradients from sp.gr. 1.09 to sp.gr. 1.16 in SW 25.1 rotor tubes and centrifuged at 25,000 rev/min for 135 minutes. The gradients were then pumped off through a u.v. monitor set at 280 nm, and collected in fractions.

### *Enzyme marker and electron micrograph studies*

Pellets prepared from the density gradient fractions were fixed in 2.5% glutaraldehyde and postfixed in Caulfields fixative (giving 1% $OsO_4$ final) for electron microscopy. Succinate dehydrogenase was estimated by the method of Ganser & Forte (1973). The 5′

nucleotidase assay was modified from Avruch & Wallach (1971). The $OD_{280}$ profiles show the lung and heart fragments to have slightly different sedimentation characteristics. Electron micrographs and enzyme marker studies confirmed that the plasma membrane rich fraction was a fairly sharp band at sp.gr. 1.115 to sp.gr. 1.12 for heart and a more diffuse band at sp.gr. 1.13 to sp.gr. 1.15 in lung. These membranes form vesicles about 1 µm diameter and little whole mitochondrial contamination is seen. The histogram in Figure 1 shows the enzyme marker distribution in one of the ventricle continuous density gradient separations.

These findings led to the routine use of sucrose step gradients to prepare quantities of plasma membranes: sp.gr. 1.09 on sp.gr. 1.136 for heart and uterus and sp.gr. 1.11 on sp.gr. 1.16 for lung.

For preparations in Krebs-Ringer buffers the tissue was homogenized or chopped in a sucrose buffer (sp.gr. 1.04) containing (mM): NaCl 122, KCl 3, $KH_2PO_4$ 0.4, $MgSO_4$ 1.2, $NaHCO_3$ 24, EGTA 0.1, theophylline 9, ICI 63,197 1.5 (a potent phosphodiesterase inhibitor—Somerville, 1973), gassed for 30 min beforehand with 95% $O_2$ and 5% $CO_2$. This buffer was also used in the density gradient steps with the appropriate concentration of sucrose and 1.5 mM NaATP in most cases. Calcium was not included due to its inhibitory effects on adenylate cyclase (Birnbaumer, 1973).

### *Chopped tissue preparations*

The tissue was excised and chopped by several passes on a McIlwain tissue chopper set at 0.5 mm, and suspended in 10 volumes of either 40 mM tris-HCl pH 7.4 containing (mM) $MgSO_4$ 6, ICI 63,197 1.5, theophylline 9, EGTA 0.1, sucrose (sp.gr. 1.04) or the Krebs-Ringer buffer as stated above. When trachea was used, individual rings were suspended in buffer in some cases; in others, the entire smooth muscle band was dissected free of cartilage and homogenized in the medium.

### *Protein estimation*

The recovery of protein at the various stages of a preparation starting from 1 g (wet wt.) of ventricle was as follows (protein estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as standard). Homogenates layered on to gradients totalled 14 mg; of this, the plasma membrane rich step gradient interface contained 2 mg, while the mitochondrial band from the continuous gradients contained 1 mg.

### *Adenylate cyclase incubations*

In chopped tissue all the enzyme systems were already present to convert 5′AMP to ATP. The cyclase incubation therefore, relied solely on endogenous ATP as substrate. In the membrane preparations exogenous ATP was added with an ATP regenerating system.

### *Chopped tissue incubation method*

Agonist (100 µl) and antagonist (100 µl) at the appropriate concentrations were mixed and preheated to 30°C in glass test tubes. The incubation was started by adding 1 ml of chopped tissue taken from a suspension which was constantly stirred at 0°C. The incubation tubes were shaken for 10 min in a waterbath set at 30°C and the reaction stopped by adding 2 NHCl (50 µl), then heated at 95°C for 10 min to extract the cyclic AMP. After adding 10 ml water and homogenizing the mixture for 10 s with a Polytron homogenizer, a 50 µl aliquot was taken for protein estimation. A 500 µl aliquot of the homogenate was neutralized to pH 7.4 with 50 µl tris (110 mM) and heated to 95°C for 7 min to precipitate the protein. After centrifuging in an Eppendorf bench centrifuge for 2 min, 50 µl of supernatant was taken for the cyclic AMP assay.

### *Membrane incubation method*

Incubation medium (200 µl 40 mM tris-HCl pH 7.4, $MgSO_4$ 6 mM, EGTA 0.1 mM, theophylline 9 mM, ICI 63,197 1.5 mM, tris ATP 1.8 mM, creatine phosphate 20 mM, 200 µg/ml creatine kinase) was added to 20 µl agonist and 20 µl antagonist at the appropriate concentrations and preheated to 30°C. The drug concentrations were randomized to minimize intra-assay variations. The reaction was started by adding 20 µl membrane suspension (3 µg lung protein or 7 µg heart protein) and incubated at 30°C for 10 min; 100 µl of 160 mM HCl was added to stop the reaction and then heated at 95°C for 6 minutes. Tris buffer (100 µl of 200 mM) was added to adjust to pH 7.4 followed by heating at 95°C for 7 minutes. After centrifuging, an aliquot of supernatant was taken for the cyclic AMP assay.

Where preparations were made in Krebs-Ringer buffer, incubations were done in Krebs-Ringer, 1.8 mM NaATP and the ATP regenerating system, and only 60 µl of 200 mM tris was required for neutralization. A zero time control was necessary to compensate the cyclic AMP assay standard curve for possible interferences from tissue or medium: HCl was added to the incubation medium which was heated to 95°C before addition of tissue or membrane.

All incubations were at 30°C and at this temperature cyclic AMP production was linear with time, at least up to 10 min, and also with protein concentration.

### *Cyclic AMP assay*

Cyclic AMP was assayed by the method of Brown, Alban, Ekins, Sgherzi & Tampion (1971) using commercial binding protein (B.D.H. Ltd., Poole, Dorset). Samples were processed in batches of 12 every 3 min in order to standardize time of contact with charcoal. Isotope counting was done by a Philips Automatic Liquid Scintillation Analyser connected on-line with a NOVA 1220 computer.

Tissue interference with the assay was eliminated by the use of zero time controls in the blanks and standard curves as described above. The use of perchloric acid for extraction of cyclic AMP and of glycyl-glycine buffers also produced interference, as did the $Ba(OH)_2/ZnSO_4$ treatment of Krishna, Weiss & Brodie (1968) when ATP was present at 1 mM or more. Crude beef adrenal binding protein had some adenylate cyclase activity which gave rise to cyclic AMP with ATP from the incubation medium, but the commercial preparation from B.D.H. was satisfactory in this respect.

### *Materials*

Propranolol (Inderal), practolol (Eraldin), atenolol (Tenormin) and halothane (Fluothane) were obtained from ICI Ltd. The other drugs and materials were obtained from various commercial sources.

## Results

Fractions isolated from sucrose density gradients of heart and lung as described above appear to have the characteristics of plasma membranes. When examined by electron microscopy they contain a large proportion of sealed vesicles with some larger membrane fragments and minimal contamination by mitochondria. They have a high content of adenylate cyclase and 5'nucleotidase and only a small amount of succinic dehydrogenase, whereas later fractions from the gradient (see Figure 1) have high succinic dehydrogenase and low cyclase and 5'nucleotidase activity. These later fractions are seen in the electron microscope to consist almost entirely of mitochondria.

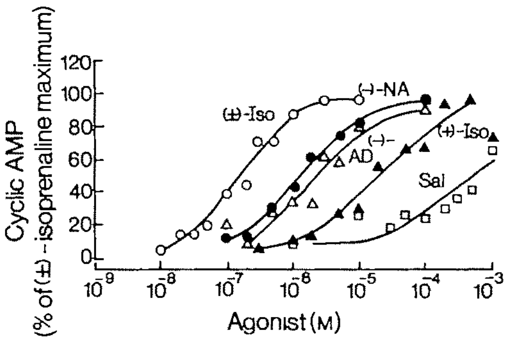

**Figure 2** Heart membranes: increase in cyclic AMP in response to (isoprenaline ((±)-Iso), ±)-Iso, (—)-noradrenaline ((—)-NA), (—)-adrenaline, ((—)-AD), (+)-isoprenaline ((+)-Iso) and salbutamol (Sal). ((±)-Isoprenaline maximum taken as 100%). Ka values, with number of experiments in brackets were: (±)-Iso $1.55 \times 10^{-7}$ (7), (—)-NA $1.23 \times 10^{-6}$ (3), (—)-AD $1.9 \times 10^{-6}$ (1), (+)-Iso $1.82 \times 10^{-5}$ (1) and Sal $1.54 \times 10^{-4}$ (3).

Uterus preparations were not characterized in as much detail as heart and lung but they were high in adenylate cyclase and electron micrographs showed membrane vesicles free of mitochondria.

Table 1 shows that pre-treatment of the rabbit with reserpine and syrosingopine increased both the basal adenylate cyclase levels and the stimulation by β-adrenoceptor agonist and sodium fluoride.

The adenylate cyclase from heart and lung responded well to hormone stimulation with a maximum increase of about 100% above basal activity. Uterus showed between 50 and 100% stimulation. Dose-response curves to (±)-isoprenaline, (—)-noradrenaline, (—)-adrenaline, (+)-isoprenaline and salbutamol, together with $K_a$ values are shown in Figures 2 and 3 for heart and lung. Since the basal level and degree of stimulation varied from one membrane preparation to another, it was necessary to express the cyclic AMP formed as a percentage of the maximum obtained with isoprenaline on the same preparation, after deducting the basal level, in order to

**Table 1** Effect of reserpine-treatment on rabbit ventricle adenylate cyclase levels

| | *Preparation I (pmol cyclic AMP per mg protein/min)* | | *Preparation II $\left(\frac{\text{pmol cyclic AMP/min}}{\mu\text{mol/h 5'nucleotidase}}\right)$* | |
|---|---|---|---|---|
| | *without reserpine* | *with reserpine* | *without reserpine* | *with reserpine* |
| Basal | 113 | 134 | 194 | 451 |
| + Isoprenaline ($10^{-4}$ M) | 219 | 310 | 310 | 821 |
| + NaF ($10^{-4}$ M) | 285 | 477 | 521 | 1679 |

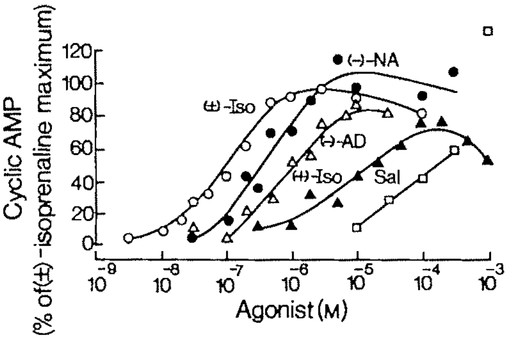

**Figure 3** Lung membranes: increase in cyclic AMP in response to (±)-isoprenaline ((±)-Iso, (—)-noradrenaline ((—)-NA, (—)-adrenaline ((—)-AD), (+)-isoprenaline ((+)-Iso) and salbutamol (Sal). ((±)-Isoprenaline maximum taken as 100%) Ka values, with number of experiments in brackets were: (±)-Iso $9.7 \times 10^{-8}$ (6), (—)-NA $4.9 \times 10^{-7}$ (5), (—)-AD $8.3 \times 10^{-7}$ (1), (+)-Iso $6.95 \times 10^{-6}$ (1) and Sal $6.94 \times 10^{-5}$ (4).

combine the results of several experiments. The $K_a$ was taken as the concentration necessary to produce 50% of the maximal stimulation obtainable with that agonist. The values for salbutamol are estimates only since at $10^{-3}$ M there was consistently an abnormally high stimulation, suggesting that a secondary effect was coming into play and making it impossible to determine a true maximum.

All agonists are more active on the lung than the heart, but the heart/lung ratios (Table 2) are very similar in all cases, suggesting that they are all acting on the same receptors. It is notable that salbutamol shows only a two-fold preference for the lung (similar to the other agonists) as compared with the 155-fold difference found between guinea-pig trachea and atria (Giles, Williams & Finkel, 1973).

To check whether some of the difference in $K_a$ values between isoprenaline and noradrenaline could be explained by the presence of a specific uptake mechanism for the natural catecholamine, the stimulation by both agonists was measured in the presence and absence of desmethylimipramine (DMI), a potent blocker of amine uptake mechanisms. Heart membranes prepared with and without reserpinization of the animal were used since reserpinization might be expected to block the storage sites and hence reduce any effect of DMI. The results (Table 3) show no significant effect either of DMI or of reserpinization and we conclude that there is no specific uptake mechanism for noradrenaline in our heart membrane preparations.

The response of the uterus to sympathomimetic agents varies with the state of oestrus and to obtain consistent responses to β-adrenoceptor stimulation it was found essential to make the animal pseudopregnant by ovariectomy followed by oestradiol and progesterone treatment as described above. Even so the dose-response curves for uterus showed more scatter than those for other tissues.

**Table 2** Comparison of agonist affinities in heart and lung membranes

| *Agonist* | *Ka heart/Ka lung* |
|---|---|
| (±)-Isoprenaline | 1.60 |
| (—)-Noradrenaline | 2.51 |
| (—)-Adrenaline | 2.29 |
| (+)-Isoprenaline | 2.62 |
| Salbutamol | 2.22 |

**Table 3** Effect of desmethylimipramine (DMI, 1 µg/ml) on adenylate cyclase activity of heart membranes prepared from reserpinized and non-reserpinized animals. For incubation conditions see text.

| | | *cyclic AMP (pmol ± s.e. mean)* | |
|---|---|---|---|
| | | *without reserpine* | *with reserpine* |
| Noradrenaline | $3 \times 10^{-7}$ M | 0.37 ± 0.01 | 0.33 ± 0.01 |
| | „ + DMI | 0.36 ± 0.01 | 0.30 ± 0.01 |
| | $3 \times 10^{-6}$ M | 0.35 ± 0.01 | 0.42 ± 0.03 |
| | „ + DMI | 0.42 ± 0.03 | 0.43 ± 0.04 |
| Isoprenaline | $3 \times 10^{-8}$ M | 0.31 ± 0.04 | 0.25 ± 0.03 |
| | „ + DMI | 0.30 ± 0.01 | 0.26 ± 0.02 |
| | $3 \times 10^{-6}$ M | 0.46 ± 0.01 | 0.36 ± 0.02 |
| | „ + DMI | 0.43 ± 0.01 | 0.33 ± 0.02 |
| | $10^{-4}$ M | 0.48 ± 0.02 | |
| Control | | 0.29 ± 0.01 | 0.16 ± 0.01 |
| | „ + DMI | 0.28 ± 0.01 | 0.16 ± 0.01 |

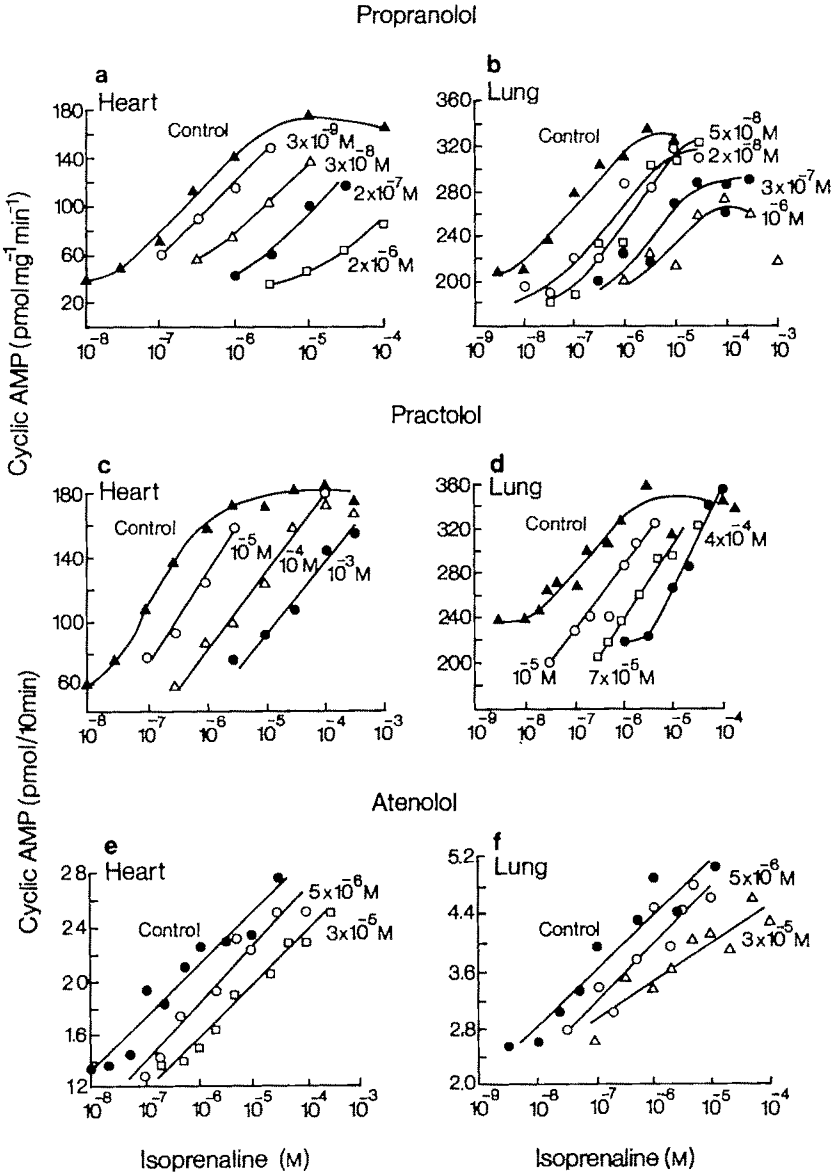

**Figure 4** (±)-Isoprenaline dose response curves to (±)-isoprenaline alone (control) and in the presence of varying amounts of propranolol (a, b), practolol (c, d) and atenolol (e, f), using heart and lung membrane preparations.

Figures 4 and 5 show the shift of the dose-response curves to isoprenaline by varying concentrations of propranolol, atenolol and practolol for heart, lung and uterus, and Figure 6 shows the corresponding Schild plots (log (dose ratio − 1) vs. log (dose)) derived from several such experiments for each drug. Similar plots for noradrenaline with propranolol and practolol are given in Figure 7 (for heart and lung only). These figures show the original uncorrected data; in order to obtain the best values for the dissociation constants ($K_b$) a statistical analysis was carried out. A standard parallel line assay procedure was used to fit parallel lines to the straight portions of the dose-response curves (determined visually) and an analysis of variance indicated that the parallel line model fitted the isoprenaline data adequately, though some deviations

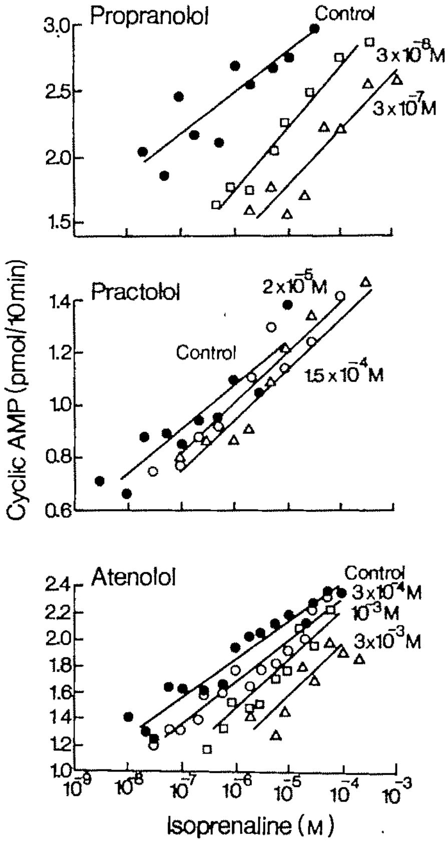

**Figure 5** (±)-Isoprenaline dose-response curves to (±)-isoprenaline alone (control) and in the presence of propranolol, practolol and atenolol, using uterus membrane preparations.

from parallelism were apparent in the noradrenaline experiments. The dose-ratio and an estimate of the variance of ln (dose-ratio) were obtained by standard techniques. Occasionally a vertical shift in the dose-response curve was apparent between different concentrations of blocking agent. In these cases the readings were reduced to a common baseline by subtracting the difference between the mean for antagonist only and the control mean.

The dose-ratio ($r$) obtained in this way were analysed by a weighted least squares analysis, regressing $\ln(r-1)$ upon $\log B$ (where $B$ = antagonist concentration). The weight attached to each value of $\ln(r-1)$ was inversely related to its estimated variance as approximated by the expression:

$$\mathrm{Var}\,(\ln(r-1)) \simeq \left(\frac{r}{r-1}\right)^2 \mathrm{var}\,(\ln r)$$

According to theory (Schild, 1957) simple competitive antagonism should result in a straight line

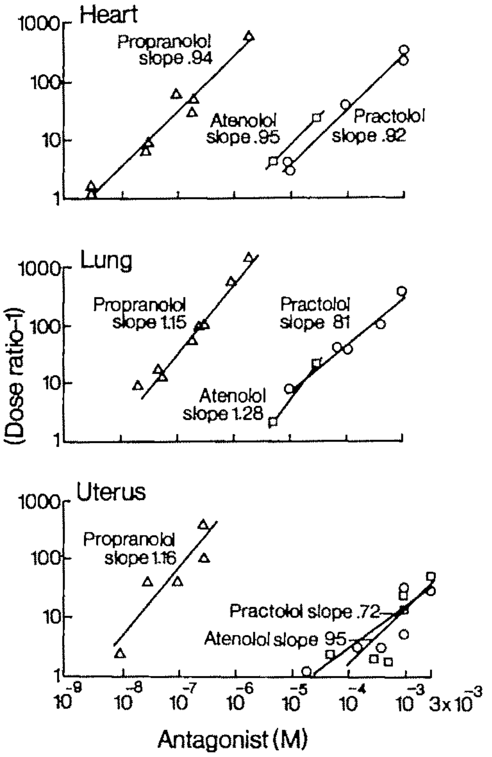

**Figure 6** Schild plots for propranolol (△), practolol (○) and atenolol (□) in heart, lung and uterus membranes, using isoprenaline as agonist.

of slope 1 and an intercept with the $x$ axis of $-\log K_b$, corresponding to the equation:

$$\log(r-1) = \log B - \log K_b$$

In no case (other than practolol and the uterus, which is discussed below) was the slope of the Schild plot significantly different from 1, so a line of slope 1 was used to calculate the $K_b$ values, which are shown in Table 4. Uncorrected Schild plots are shown in Figures 6 and 7. Since the data for whole lung and heart revealed no significant degree of cardioselectivity, we endeavoured to separate the bronchial tree from the spongy tissue of the lung by mechanical combing and examined the responses of both fractions to isoprenaline and atenolol. We also used preparations of tracheal rings and homogenates of tracheal smooth muscle. The Schild plots of Figure 8 show that the dose-ratios for both fractions of lung fall on the same line as for whole lung, while the response of the trachea is intermediate between lung and uterus. Histological examination of these tissues showed that the proportion of smooth muscle in the 'bronchial tree' fraction was very small and not too different from the 'spongy tissue' and that the smooth muscle of the

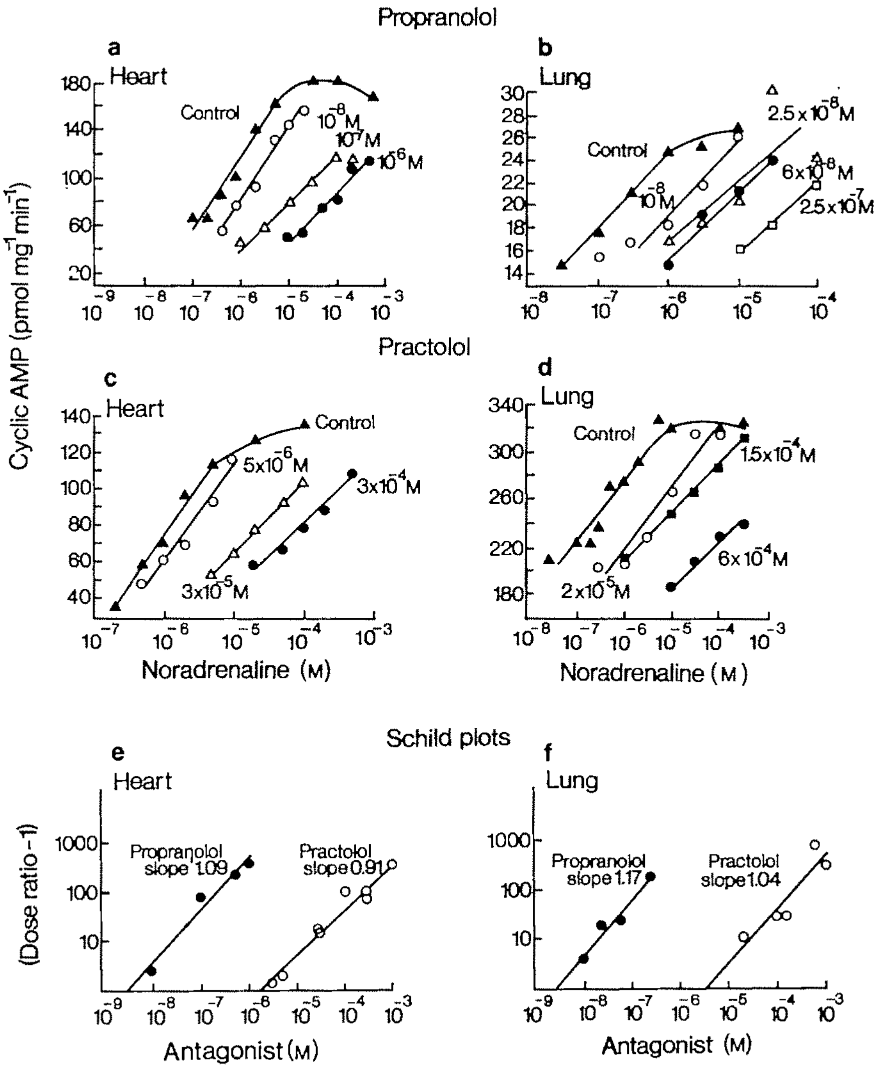

**Figure 7** (−)-Noradrenaline dose response curves to noradrenaline alone (control) and in the presence of varying amounts of propranolol (a, b) and practolol (c, d) together with Schild plots (e, f), using heart and lung membrane preparations.

trachea still retained its inner lining of epithelial cells so that the possibility of a mixed response existed. The situation in uterus is quite otherwise and there is clear demonstration of a difference in dissociation constant (Figures 6 and 8, Table 4). The slope of the Schild plot for practolol is significantly less than 1, which suggests either that the inhibition is not strictly competitive or that the interaction of practolol with the uterus receptor is not 1:1—in both cases reinforcing the conclusion that the uterus receptor is different from the heart.

Some experiments were also carried out with chopped tissue, and at the same time the effects of preparing tissues in Krebs-Ringer buffer instead of the tris buffer which had been used in all previous experiments was compared, since a number of reports in the literature suggest that tris may have harmful effects on membranes and even result in the loss of hormone stimulation from brain cyclase preparations (Chasin, Mamrak & Samaniego, 1974). Figures 9 and 10 show dose-response curves to isoprenaline in chopped heart and lung (prepared in Krebs-Ringer) in the presence and absence of $10^{-4}$ M practolol, and Table 5 summarizes the $K_a$ values for isoprenaline and $K_b$ values for practolol obtained in two experiments

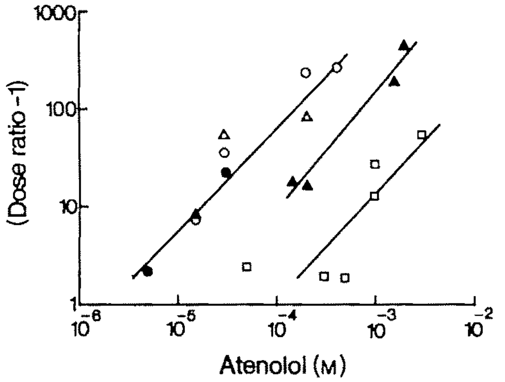

**Figure 8** Schild plots for atenolol in trachea (▲), uterus (☐) and various lung preparations using isoprenaline as agonist. (●), whole lung membranes; (○), homogenate of lung spongy tissue and (△) homogenate of lung bronchial tree.

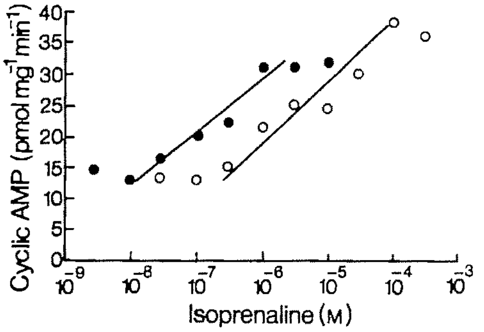

**Figure 9** Chopped heart tissue (in Krebs-Ringer). Dose-response curves to isoprenaline alone(●) and in the presence of $10^{-4}$ M practolol (○).

with tissues prepared in Krebs-Ringer and one in which tris buffer was used. The results show that tris is without important effect and that the $K_a$ values obtained with chopped tissue preparations do not differ from those obtained with membranes.

Similar $K_a$ and $K_b$ values were obtained from freshly prepared membranes and from membranes stored for some time in liquid nitrogen. Taken together these factors lead us to believe that the $\beta$-adrenoceptors and their linkage to adenylate cyclase have not been significantly perturbed in the process of membrane preparation and storage, and are, therefore, representative of $\beta$-adrenoceptors in the whole organ.

## Discussion

The cardioselectivity of practolol and atenolol in isolated organs has been clearly demonstrated by Barrett, Carter, Fitzgerald & Le Count (1973) and

**Table 4** Antagonist K values (mM) determined on membranes. (Calculated from corrected data and Schild plots of slope 1—see text).

| | | | $K_b$ (nM ± s.e. mean) | $K_b$ relative to heart |
|---|---|---|---|---|
| *a. Isoprenaline as agonist* | | | | |
| | Propranolol | Heart | 3.4 ± 0.5 | 1 |
| | | Lung | 2.83 ± 0.32 | 0.85 |
| | | Uterus | 1.5 ± 0.6 | 0.45 |
| | Practolol | Heart | 3200 ± 400 | 1 |
| | | Lung | 2100 ± 390 | 0.65 |
| | | Uterus* | (54,000) | (17) |
| | Atenolol | Heart | 1300 ± 600 | 1 |
| | | Lung | 1600 ± 400 | 1.23 |
| | | Uterus | 67,000 ± 20,000 | 51.5 |
| *b. Noradrenaline as agonist* | | | | |
| | Propranolol | Heart | 2.3 ± 0.5 | 1 |
| | | Lung | 1.7 ± 0.3 | 0.74 |
| | Practolol | Heart | 2600 ± 300 | 1 |
| | | Lung | 2200 ± 700 | 0.84 |

* Schild plot slope significantly < 1

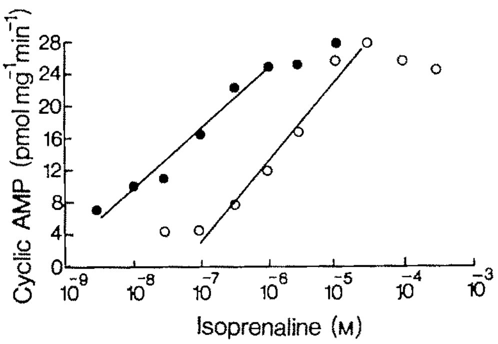

**Figure 10** Chopped lung tissue (in Krebs-Ringer). Dose-response curves to isoprenaline alone (●) and in the presence of $10^{-4}$ M practolol (○).

Table 6 shows the $K_b$ values for atrium and trachea derived from these authors' pA2 values (in some cases updated to include more recent experiments from the same laboratory—personal communication from Mr J. Carter and Dr J. Wale). Selectivity could be due to differences in one or more of the following factors: 1 the $\beta$-adrenoceptors; 2. non-specific binding of the drug; 3. the presence of diffusion barriers to the $\beta$-adrenoceptor sites.

Factors 2 and 3 could act in the whole organ to reduce the effective concentration of a drug at the $\beta$-adrenoceptor of one tissue relative to the other and make it more difficult for the drug to reach a true equilibrium with the receptor. Our expectation was that these factors would be considerably reduced in membrane preparations and hence that $K_b$ values obtained with these preparations would be a truer measure of receptor interaction. In the event, the similarity of values obtained for practolol in both membranes and chopped tissue suggests that there is no question of equilibration in the tissue being incomplete as compared with the membrane. The fact that the $K_b$ for propranolol measured on the cyclic AMP response of heart membranes ($3.4 \pm 0.5 \times 10^{-9}$ M) is very close to that measured on the chronotropic response of whole atrium ($4.9 \pm 0.61 \times 10^{-9}$M) reinforces the view, expressed above, that the receptors have not been greatly altered during preparation of the membranes. We have, therefore, to look elsewhere for an explanation of the lack of selectivity shown by practolol and atenolol in heart v. lung membranes and the most likely reason lies in the multiplicity of cell types to be found in lung. Histological examination shows a relatively small proportion of bronchial smooth muscle cells and if these were sensitive to $\beta_2$-adrenoceptor stimulation it is conceivable that their response in terms of cyclic AMP could be swamped by a large excess of $\beta_1$ responding cells (possibly secretory). Neither mechanical combing of the tissue nor density gradient centrifugation of the membranes appear to produce any separation of these types. Further work on characterizing the adrenergic response of the cell types involved is in progress but in the meantime we have to say that the predominant response of the lung to adrenoceptor agonists, in terms of cyclic AMP, is $\beta_1$, though this is not necessarily true of bronchial smooth muscle. The contrary results found by other authors (Burges & Blackburn, 1972; Lefkowitz, 1975) can be traced to the

**Table 5** Comparison of $K_a$ values for isoprenaline and $K_b$ for practolol obtained with chopped tissue in tris buffer (1 expt.) and Ringer (2 expts.). For details see text.

| | *Buffer* | *Ka for Isoprenaline (μM)* | *Kb for Practolol (μM)* |
|---|---|---|---|
| Heart | *Tris* | 0.24 | 4.2 |
| | *Ringer (1)* | 0.103 | 2.0 |
| | *Ringer (2)* | 0.11 | 3.3 |
| Lung | *Tris* | 0.035 | 4.3 |
| | *Ringer (1)* | 0.084 | 5.0 |
| | *Ringer (2)* | 0.056 | |

**Table 6** $K_b$ values (nM ± s.e. mean) determined on isolated tissues. Isoprenaline used as agonist. (Data adapted from Barrett *et al.* (1973) updated by personal communications from Mr J. Carter and Dr J. Wale)

| | *Atrium** | *Trachea†* | *Ratio Trachea/Atrium* |
|---|---|---|---|
| Propranolol | 4.9 ± 0.61 | 21.5 ± 6.0 | 4.4 |
| Practolol | 324 ± 7 | 24,320 ± 4180 | 75.1 |
| Atenolol | 53.7 ± 14 | 6090 ± 810 | 113.4 |

* Spontaneous beating rate of guinea-pig right atrium
† Relaxation of guinea-pig tracheal smooth muscle

inaccuracies of comparing $ED_{50}$ values for antagonists. These values, estimated from dose-response curves at a single, fixed, agonist concentration, do not allow for differences in agonist $K_a$ between tissues and are subject to considerable error depending on the agonist concentration chosen. The use of dissociation constants calculated from Schild plots, as in the present study, is free from these disadvantages.

The same two compounds, practolol and atenolol, show a much lower potency on uterus membranes and we conclude that this represents a real difference in receptor type. The intermediate position of the trachea presumably indicates a mixed $\beta_1$- and $\beta_2$-adrenoceptor population. The selectivity ratios found in membranes, however, are considerably lower than those found in the whole organ system (Tables 4 and 6) and experiments with a wider series of compounds (Coleman & Somerville, in preparation) show that the difference varies in a systematic manner with partition coefficient. This suggests that different receptor types alone are not sufficient to account for selectivity, but that distribution of the drug in the micro-environment of the receptor may also have a part to play.

We are indebted to Mrs C. O'Neill and Mr C.D. Adams for excellent technical assistance and to Dr R.A. Ferguson for the statistical calculations. This work forms part of a Ph.D. thesis to be submitted by Mr A.J. Coleman to the Council for National Academic Awards.

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*(Received April 23, 1976.*
*Revised July 12, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 95–100

# NICOTINE STIMULATES PROSTAGLANDIN FORMATION IN THE RABBIT HEART

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1 Rabbit isolated hearts were perfused according to Langendorff with Tyrode solution. Prostaglandin-like substances (PLS) in the effluent were purified and assayed on the rat stomach strip. Noradrenaline (NA) in the effluent was assayed fluorimetrically.

2 Infusion of nicotine (1 μM–50 μM) caused a dose-dependent, brief increase, from 1.2 ± 0.4 to maximally 8.3 ± 2.1 ng/min, in the outflow of PLS from the heart. The increase was abolished by pretreatment of the heart with indomethacin.

3 Activation of nicotinic receptors in the heart with acetylcholine (ACh, 200 μM) in the presence of atropine (1 μM) also elicited an increase in the release of PLS. This release was smaller than that caused by nicotine.

4 Nicotine (50 μM) and ACh (200 μM) in the presence of atropine (1 μM) each caused a pronounced but brief release of NA into the effluent. There was no evident correlation between the ability of the drugs to cause release of PLS on the one hand, and NA on the other.

5 It is concluded that nicotine acts as a direct stimulus for the synthesis of prostaglandins in the rabbit heart.

## Introduction

The synthesis of prostaglandins in tissues, as evidenced by their appearance in the venous effluent, can be raised above the basal level by a variety of agents. Not only do drugs like adrenaline, noradrenaline and angiotensin stimulate prostaglandin synthesis (Davies, Horton & Withrington, 1968; Gilmore, Vane & Wyllie, 1968; McGiff, Crowshaw, Terragno & Lonigro, 1970a; Junstad & Wennmalm, 1973; Minkes, Douglas & Needleman, 1973), but also metabolic intervention such as hypoxia and ischaemia (McGiff, Crowshaw, Terragno, Lonigro, Strand, Williamson, Lee & Ng, 1970b; Block & Vane, 1973; Kent, Alexander, Pisano, Keiser & Cooper, 1973; Wennmalm, Pham-Huu-Chanh & Junstad, 1974).

Recently it has been demonstrated, in the rabbit isolated heart and in human forearm muscle, that nicotinic acid is a potent stimulator of prostaglandin synthesis (Kaijser & Wennmalm, 1976; Kaijser, Eklund & Wennmalm, 1976). It was therefore of interest to study whether nicotine, which has certain structural similarities with nicotinic acid, is likewise able to stimulate prostaglandin synthesis. In a preliminary report (Wennmalm & Junstad, 1976) such an action was in fact demonstrated. In the present paper further results are described concerning the stimulatory effect of nicotine on rabbit myocardial prostaglandin formation.

## Methods

### *Rabbit heart perfusion*

Rabbits of mixed strains and either sex were used for the study. The weight of the animals varied from 1.2 to 2.4 kg. After a blow on the head, the animal was exsanguinated by cutting the left carotid artery. The heart was rapidly removed and transferred to the perfusion apparatus, where it was perfused according to Langendorff with Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, MgCl 1.0, $NaHCO_3$ 12, $NaH_2PO_4$ 0.4 and glucose 5.6. The solution was aerated continuously with 5% $CO_2$ in $O_2$. The pH of the solution was 7.4–7.5. The perfusion pressure was 6 kPa and the temperature was kept at 37°C. The apex of the heart was connected to a strain gauge transducer, and heart rate and contractile force were recorded on a Grass Model 5 D Polygraph.

### *Analysis of prostaglandin-like substances and noradrenaline*

During the experiments the effluent from the heart was collected in 5 min periods. It was immediately chilled and acidified to pH 3–4. A 40 ml portion was separated for subsequent NA analysis (see below). The remaining effluent was passed through a column containing Amberlite XAD–2 and the lipids were

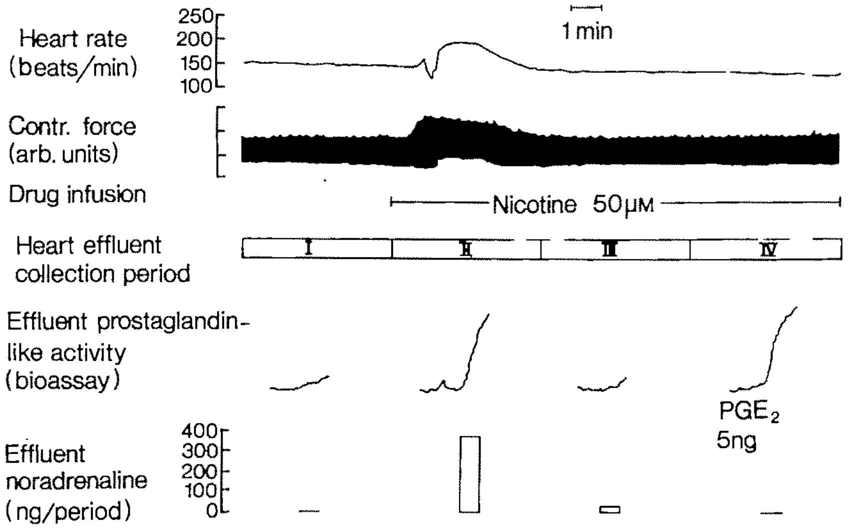

**Figure 1** Perfused rabbit heart. The upper two tracings show the heart rate and the contractile force at rest and during infusion of nicotine. The lower tracing demonstrates the contractions of the rat stomach strip exposed to lipid extracts of the effluent from the heart, collected in 5 min periods as indicated by the blocks I–IV. The last bioassay tracing shows the contraction of the bioassay organ when a standard amount of prostaglandin $E_2$ ($PGE_2$) is added. The lower panel demonstrates the content of noradrenaline in the effluent, collected in 5 min periods as indicated by the blocks I–IV.

eluted in ethanol (Keirse & Turnbull, 1973). After evaporation the residue was dissolved in 0.9% w/v NaCl solution (saline) and assayed on the rat stomach strip (Vane, 1957) against known amounts of prostaglandin $E_2$. The assay tissue was kept in a 5 ml organ bath containing gassed and warmed Tyrode solution as above. To the solution were added phentolamine (0.7 μM), propranolol (0.8 μM), atropine (0.1 μM), methysergide (0.6 μM), and diphenhydramine (0.7 μM), in order to block activity in the strip due to the presence of NA, adrenaline, acetylcholine, 5-hydroxytryptamine, or histamine in the lipid extract from the heart effluent. The method permits outflow rates of less than 1 ng/min of prostaglandin $E_2$ equivalents to be assayed accurately. The recovery of external standards of prostaglandin $E_2$ was 53 ± 4% ($n = 20$). The outflow figures for PLS given here are corrected for losses during purification by the use of the present recovery method.

NA was adsorbed on alumina and assayed fluorimetrically by the trihydroxy-indole method as described by Euler & Lishajko (1961). The recovery of external standards of NA was 96 ± 4% ($n = 22$). The outflow figures for NA are not corrected for losses during purification.

### *Drugs*

Nicotine was obtained in pure liquid form from the chemical analysis laboratory at the Swedish Tobacco Company. It was kept in the dark and under nitrogen until used. It was dissolved in Tyrode solution just before the experiment and infused into the perfusion system via a cannula close to the aorta.

Acetylcholine (Hoffman-La Roche & Co.) was dissolved in Tyrode solution before the experiment and infused via the aortic cannula to produce a final concentration of 200 μM.

Atropine sulphate (Sigma Chemical Co.) was added directly to the Tyrode solution, at a concentration of 1 μM.

Indomethacin (Merck, Sharpe & Dohme) (as the pure substance) was dissolved in ethanol. Immediately before the experiment it was added to the Tyrode solution to produce a final concentration of 50 μM.

### *Calculations*

Values in the text and table are given as mean ± s.e. mean. Figures in brackets indicate the number of experiments. Student's *t*-test has been used for statistical analysis of differences between the outflow figures of PLS obtained in the different series.

## Results

The spontaneous beating frequency of the hearts in the perfusion apparatus ranged from 120 to 160 beats/minute. During sustained perfusion in the absence of drug administration, this spontaneous rate generally fell slightly. The contractile force also faded slightly during the course of the experiments. The perfusion flow ranged from 30 to 45 ml/min and changed insignificantly during the experiments. The basal effluent content of PLS ranged from 1.2 to 2.8 ng/minute. No NA was found in the effluent during basal conditions.

### *Infusion of nicotine (1–50 μM)*

The lowest concentration of nicotine (1 μM) failed to affect the mechanical performance of the heart. The overflow of PLS in the effluent remained at the basal level. No NA release was observed.

At a concentration of 10 μM, nicotine induced increases in the heart rate (by 24 ± 6 beats/min) and the contractile force (by 20–30%). The PLS concentration in the effluent remained at the basal level (2.2 ± 0.5 ng/min). There was a substantial release of NA into the effluent, amounting to 238 ± 29 ng during the 5 min collection of effluent.

The highest nicotine concentration (50 μM) elicited marked changes in the mechanical performance of the heart. After a brief initial decrease (Figure 1), heart rate increased 61 ± 6 beats/minute. The contractile force was almost doubled. The outflow of PLS increased markedly, to 8.3 ± 2.1 ng/minute. There was also a release of NA into the effluent, amounting to 337 ± 43 ng during the infusion period. In spite of sustained nicotine exposure, the mechanical performance and the effluent's content of PLS and NA gradually returned towards the basal level, complete return to base-line conditions being obtained after about 5 min of drug exposure.

When indomethacin was infused before and during the exposure to nicotine (50 μM), the increases in heart rate and contractile force were mainly the same as in the absence of indomethacin. The release of NA (872 ± 166 ng) during nicotine exposure was significantly ($P < 0.01$) higher than in the absence of indomethacin (Figure 2). The basal outflow of PLS was completely abolished, and only insignificant amounts were found in the effluent during nicotine exposure.

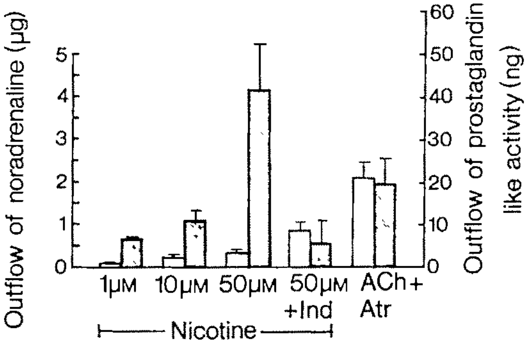

**Figure 2** Perfused rabbit heart. The columns represent the outflow of noradrenaline (NA, open columns) and prostaglandin-like substance (PLS, hatched columns) during the first 5 min following exposure to various concentrations of nicotine, in the absence or presence of indomethacin (50 μM), or to acetylcholine (200 μM) in the presence of atropine (1 μM). Note the marked difference between the ability of nicotine (50 μM) and acetylcholine plus atropine to induce liberation of PLS on the one hand and NA on the other.

### *Infusion of acetylcholine (200 μM) in the presence of atropine (1 μM)*

The mechanical response of the heart to exposure to ACh (200 μM) in the presence of atropine (1 μM) differed from that elicited by nicotine both quantitatively and qualitatively. Only in some

**Table 1** Outflow of prostaglandin-like substances, expressed as equivalents of prostaglandin $E_2$, in ng/min during four consecutive 5 min periods of effluent collection from the perfused rabbit heart.

| *A. Nicotine* | I | II | III | IV |
|---|---|---|---|---|
| 1. II–IV: 1 μM | 1.4 ± 0.2 (3) | 1.3 ± 0.2 (3) | 0.9 ± 0.5 (3) | 0.6 ± 0.6 (3) |
| 2. II–IV: 50 μM | 1.2 ± 0.4 (7) | 8.3 ± 2.1 (7)** | 3.1 ± 1.0 (7) | 1.0 ± 0.4 (7) |
| 3. I: 10 μM; II–IV: 50 μM | 2.2 ± 0.5 (5) | 6.8 ± 0.7 (5)** | 2.9 ± 1.1 (5) | 2.5 ± 1.4 (5) |
| 4. II–IV: 50 μM<br>I–IV: indomethacin 50 μM | 0 (2) | 1.1 ± 1.1 (2) | 0.4 ± 0.4 (2) | 0 (2) |
| *B. Acetylcholine (+ atropine 1 μM)* | | | | |
| 1. II–IV: 200 μM) | 2.8 ± 0.7 (5) | 3.9 ± 1.2 (5) | 2.0 ± 0.5 (5) | 2.6 ± 0.9 (5)* |

Values are expressed as mean ± s.e.; figures in parentheses indicate number of experiments. ** Indicates that the value is significantly ($P < 0.01$) higher than the corresponding value in the preceding effluent collection period.

experiments was an increase observed in the heart rate and this was both small and brief. The inotropic response differed between the experiments, being absent in some and very pronounced in others. Roughly, the increase in contractile force was correlated to the effluent's content of NA. There was a moderate but insignificant and only initial increase in the effluent's content of PLS (Table 1). The most prominent effect of exposing the heart to ACh and atropine was that large quantities of NA appeared in the effluent (2091 ± 380 ng) (Figure 2). The outflow of NA from the organ during exposure to this drug combination by far exceeded that induced by nicotine, in the absence as well as in the presence of indomethacin. The release of NA was brief and decreased exponentially during sustained exposure of the heart to ACh plus atropine.

## Discussion

The present experiments clearly demonstrate that the addition of nicotine to the medium perfusing the isolated heart of the rabbit elicits a release of PLS into the venous effluent of the organ. As it is well known that tissues do not store more than minute amounts of PLS, the increased concentration in the effluent seems to reflect an increased formation in the heart. Nicotine's stimulation of PLS synthesis was dose-dependent, rising steeply in the drug concentration range 1 μM to 50 μM. The mechanism behind the increased formation of PLS might be either an augmented mobilization of the precursor fatty acid from the phospholipid stores, or an increased synthetase activity. It was beyond the scope of the present investigation to elucidate which of these mechanisms in fact operates during nicotine-stimulated formation of PLS, but by analogy with other tissues an increased availability of precursor seems likely (cf. Oates, Seyberth, Oelz, Danon & Sweetman, 1975). During the sustained administration of nicotine, the effluent's concentration of PLS gradually returned to the basal level. It has been shown earlier (Wennmalm, 1975) that larger amounts of PLS than those observed here may be released into the effluent of the rabbit isolated heart. Exhaustion of the precursor stores should consequently not have limited the continued formation of PLS during sustained myocardial exposure to nicotine. The decline of PLS in the effluent therefore probably reflects a decreased synthetase activity, although the possibility of an increased degradation cannot be ruled out completely. These experiments consequently demonstrate a dual action of nicotine on the myocardial PLS synthesis, stimulatory as well as inhibitory.

In the mammalian heart, the post-ganglionic adrenergic neurones contain nicotine receptors which on activation mediate a release of NA from the intraneuronal transmitter stores. Such a release of NA was observed in the present experiments during administration of nicotine in the dose range of 10 μM–50 μM, as reflected by the appearance of NA in the effluent. The positive chronotropic and inotropic response of the heart upon drug exposure was probably due to activation of adrenoceptors by the transmitter released. As shown by Löffelholz (1970), the NA release in the rabbit heart by nicotine is 'explosive', and subject to sudden auto-inhibition during continued drug administration. This was confirmed in the present study. Since the effluent's concentration of PLS also decreased, in spite of sustained exposure to nicotine, it seemed that the releases of NA and PLS from the heart might be causally related. A logical basis for such a relationship exists, since it has been shown that sympathetic nerve stimulation elicits parallel NA and prostaglandin E releases from the rabbit heart (Wennmalm & Stjärne, 1971); furthermore, noradrenaline infused into the rabbit isolated heart elicits a release of prostaglandin E into the venous effluent of the organ (Junstad & Wennmalm, 1973). Such a causal relationship would imply that the stimulation of PLS synthesis in the present experiments was not primarily due to the action of nicotine, but a consequence of the vast amounts of NA released by the drug. In order to test this hypothesis, a series of experiments was performed in which NA release was elicited via the activation of nicotine receptors by the infusion of ACh (200 μM) in the presence of atropine (1 μM) (cf. Löffelholz, 1970). It was found that this drug combination causes a more pronounced release of NA than nicotine. Even in these experiments a sudden auto-inhibition of the release appeared, indicating a common mechanism behind the NA release by nicotine and by ACh in the presence of atropine. However, the release of PLS was stimulated only moderately by ACh in the presence of atropine, in spite of the marked NA release. The low PLS release with ACh plus atropine, compared with that caused by nicotine, suggests that the NA released was responsible only in part, if at all, for the increased formation of PLS. This leads to the conclusion that nicotine as such stimulates the synthesis and subsequent release of PLS in the rabbit myocardium. Furthermore, this effect of nicotine seems to be distinct from the drug's pharmacological actions, which are generally referred to the activation of 'nicotinic' receptors.

Although the PLS in the myocardial effluent was not identified completely, there are several reasons for regarding it as prostaglandin E. Firstly, prostaglandins are almost the only smooth muscle stimulating compounds which follow the purification scheme used. Secondly, the rat stomach strip, suspended in a medium containing inhibitors for the activity of adrenaline, NA, ACh, 5-hydroxytryptamine, and histamine, seems to be specific for the assay of prostaglandin E. Thirdly, the increase in PLS caused by nicotine was inhibited by pretreatment of the heart with indomethacin, a well-known prostaglandin synthesis inhibitor (Vane, 1971). Finally, the PLS in the effluent had the same biochemical and biological properties as the prostaglandin E which has been shown to be released from this preparation (Wennmalm, 1971; 1975).

A side observation of some interest was made in the present investigation, namely that the nicotine-induced release of NA was greater in the presence than in the absence of indomethacin. This suggests that inhibition of PLS formation leads to facilitation of the nicotine-induced release of NA. It is well-known that the release of NA induced by sympathetic nerve depolarization is inhibited by exogenous prostaglandin E in several tissues and species (cf. Hedqvist, 1970). Furthermore, it has been shown that inhibition of the endogenous synthesis of prostaglandin in the rabbit heart leads to facilitation of the transmitter release induced by sympathetic nerve stimulation (Samuelsson & Wennmalm, 1971). The current observation, that inhibition of the endogenous synthesis of PLS is followed by an increased nicotine-induced release of NA, suggests that this drug also elicits transmitter liberation by a process that is inhibited by endogenous prostaglandins.

To summarize, the current investigation has demonstrated a stimulatory effect of nicotine on the myocardial prostaglandin synthesis. The effect is brief and appears to be separated from activation of the conventional 'nicotinic' receptors.

Nicotine is probably the most common of the drugs used by man. In view of the current observations, it seems of interest to elucidate whether the concentration of nicotine in plasma from smoking subjects may become sufficiently high to stimulate the synthesis of prostaglandins. If so, the synthesis of the potentially hazardous, recently discovered prostaglandin analogue, thromboxane (Hamberg, Svensson & Samuelsson, 1975) may be increased as well. Thromboxane induces platelet aggregation; it therefore seems essential to study whether a relation exists between nicotine and myocardial thrombus formation.

This investigation was supported by the Swedish Tobacco Company, and by the Swedish Medical Research Council, project 04X-4341.

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*(Received May 19, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 101–106

# ACCUMULATION OF RADIOACTIVE CARDIAC GLYCOSIDES BY VARIOUS BRAIN REGIONS IN RELATION TO THE DYSRHYTHMOGENIC EFFECT

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**1** Ouabain was administered at a loading dose of 3 μg/kg followed by an infusion at a rate of 1 μg $kg^{-1}$ $min^{-1}$ in order to produce severe dysrhythmia in dogs within 60 minutes. Similarly, digitoxin at a loading dose of 9 μg/kg followed by an infusion at a rate of 3 μg $kg^{-1}$ $min^{-1}$ was administered to compare its effect with that of ouabain.

**2** During the 60 min experimental period, the plasma concentrations gradually rose with the continuous infusion of these drugs. However, in comparison to the 60 min plasma value of 119 ± 20 pmol/ml for ouabain and 177 ± 68 pmol/ml for digitoxin, the cerebrospinal fluid (CSF) concentrations for these drugs at this time were less than 5 pmol/ml.

**3** Upon termination of the experiment at 60 min it was found that kidney, liver, heart, adrenal, and the non-neural tissue in the brain such as pituitary and choroid plexus concentrated ouabain and digitoxin to give high tissue to plasma ratios. However, various neural areas of the brain (cerebellum, mesencephalon, hypothalamus, pons, and medulla) showed no preferential localization or uptake of these two glycosides.

**4** Concentration of ouabain and digitoxin by the choroid plexus does not seem to affect the ionic composition of the CSF.

**5** It was concluded that sampling the large areas of neural tissue listed above could provide no evidence for local accumulation of digitalis glycosides that might account for a central nervous system origin of digitalis-induced cardiac arrhythmias.

## Introduction

Previous reports have drawn attention to the possibility that cardiac glycosides may produce effects on the heart through the central nervous system (CNS), apart from any direct action upon the myocardium. Recently, results implicating the CNS in the dysrhythmogenic effects of cardiac glycosides have been reported by Gillis and colleagues (Gillis, 1969; Gillis, Dionne & Standaert, 1972a; Gillis, Raines, Sohn, Levitt & Standaert, 1972b). By simultaneously recording sympathetic and phrenic nerve activity together with the ECG, the above workers demonstrated a relationship between the enhanced neuronal activity and cardiac disorders following systemic injection of digitalis. We felt that these studies of Gillis (1969) and recent claims of Garan, Smith & Powell (1974) were sufficiently impressive to require careful evaluation of the CNS as a possible site of digitalis-induced dysrhythmia. Thus, we undertook a systematic evaluation to determine: (1) the rate of appearance of systemically administered cardiac glycosides, namely ouabain and digitoxin, in the cerebrospinal fluid (CSF); (2) the uptake of these cardiac glycosides in various brain areas; and (3) the effect of these cardiac glycosides upon the ionic composition of the CSF.

## Methods

Experiments were conducted on male and female mongrel dogs anaesthetized with a loading dose of 1 ml/kg $\alpha$-chloralose (7.5%) and urethane (50%). The anaesthesia was supplemented, if necessary, by 0.1 ml/kg doses of this solution. Cardiovascular measurements including left intraventricular pressure (LVP), the rate of change of LVP ($dp/dt$), aortic blood pressure and lead II ECG were continuously monitored on a Beckman dynograph. The LVP was recorded by retrograde insertion of a catheter through the left femoral artery into the lumen of the left ventricle. The end of the catheter was connected to a Beckman miniature pressure transducer and the ventricular pressure wave was recorded on one channel of the polygraph. The first derivative of the output from the LVP was obtained directly from the ventricular pressure signal by means of a resistance-capacitance (R-C) differentiating circuit and displayed on another channel of the polygraph. Aortic pressure was recorded in the thoracic aorta by inserting another catheter through the right femoral artery. This catheter was also connected to a Beckman pressure transducer by means of a three-way stop-cock.

Arterial blood samples were collected serially through the aortic catheter. A 22 gauge spinal needle was inserted into the cisterna magna for serial collection of CSF samples. After control samples of blood and CSF were taken, tritium labelled ouabain or digitoxin was administered intravenously. The dose of ouabain consisted of an injection of a 3 µg/kg loading dose followed by infusion at a rate of 1 µg kg$^{-1}$ min$^{-1}$. In the case of digitoxin, a loading dose of 9 µg/kg was followed by a 3 µg kg$^{-1}$ min$^{-1}$ infusion. Unlabelled ouabain and digitoxin were mixed with small amounts of tritiated ouabain and digitoxin of high specific activity. Depending on the weight of the dog, approximately 50–60 µCi of tritiated drug was administered per experiment. Radioactive ouabain (20 µCi/µg) and digitoxin (40 µCi/µg) were obtained commercially as the randomly labelled product (New England Nuclear Corp.). Drugs were infused with a Desaga peristaltic pump through a catheter inserted into the left femoral vein.

At the termination of each experiment, 30–50 mg samples from left ventricle of the heart, kidney, liver, and adrenal gland were obtained along with brain samples to determine their radioactive content. The brain areas dissected out for radioactive counting were: medulla, pons, mesencephalon, cerebellum, visual cortex, hypothalamus, pituitary, and the choroid plexus of the lateral ventricles. Radioactivity in the tissue, blood plasma, and CSF samples was determined by a liquid scintillation spectrometer. Commercially available phase combining system (Amersham–Searle) was used as the scintillation fluid. All samples were counted for a period of time sufficient to limit the counting error to 5% or less.

For measurement of the plasma and CSF sodium, potassium, calcium, and magnesium, aliquots were analyzed with an atomic absorption spectrophotometer. The concentration of each ion was expressed in mmol/litre of the respective body fluid.

## Results

### *Electrophysiological effects manifested by dysrhythmogenic doses of cardiac glycosides*

In all dogs treated with ouabain, severe ventricular tachycardia was observed within 60 min, at which time the experiment was terminated. In some experiments, dysrhythmia appeared after 15 min of infusion and was sustained throughout but in others, the onset was later. In contrast, digitoxin did not induce severe dysrhythmia during the one hour infusion period despite the dosage being three times as great as that of ouabain.

### *Plasma and CSF cardiac glycoside concentration*

After administration of the loading dose and initiation of ouabain and digitoxin infusion, plasma concentrations of the drugs gradually rose but did not plateau during the 60 min experimental period. The average plasma and CSF concentrations of ouabain at 60 min were 119 ± 20 and 2.9 ± 1.2 pmol/ml, respectively (Tables 1 and 2). Digitoxin plasma levels were somewhat higher, but the CSF concentration of digitoxin was similar to that of ouabain. The plasma/CSF ratio for ouabain and digitoxin showed no significant difference and appeared to stabilize at approximately 40:1 and 50:1 respectively.

### *Ouabain and digitoxin concentration in brain regions and in other tissues*

No neuronal brain region studied, including the hypothalamus, pons, and medulla was found to

**Table 1** The concentrations of [$^3$H]-ouabain and [$^3$H]-digitoxin in plasma and cerebrospinal fluid (CSF) at various time intervals following intravenous administration of these drugs

| | *Plasma (pmol/ml)* | | *CSF (pmol/ml)* | |
|---|---|---|---|---|
| | *Ouabain* | *Digitoxin* | *Ouabain* | *Digitoxin* |
| *Time (min)* | *mean ± s.e.* (n=*6)* | (n=*3)* | *mean ± s.e.* (n=*6)* | (n=*3)* |
| 5 | 46 ± 9 | 54 ± 15 | — | 0.8 ± 0.7 |
| 10 | 50 ± 12 | — | 0.9 ± 1.0 | — |
| 15 | — | 88 ± 27 | — | 0.5 ± 0.5 |
| 20 | 65 ± 15 | — | 1.2 ± 1.3 | — |
| 30 | 68 ± 12 | 121 ± 36 | 2.3 ± 2.8 | 2.0 ± 1.4 |
| 40 | 93 ± 18 | — | 2.1 ± 1.1 | — |
| 45 | — | 152 ± 52 | — | 2.4 ± 1.5 |
| 60 | 119 ± 20 | 177 ± 68 | 2.9 ± 1.2 | 3.2 ± 3.2 |

contain more than a small amount of ouabain. The ouabain content was 10% or less of the plasma concentration (Table 2). However, the pituitary and choroid plexus, concentrated ouabain to a great extent as reflected by 7.1 and 1.74 fold tissue: plasma binding ratios. Localization of ouabain in the visceral organs and skeletal muscle was generally high, the kidney accumulating ouabain to the greatest extent. The heart also accumulated a substantial concentration of ouabain and lesser levels were found in the adrenal gland and liver. Skeletal muscle accumulated the least on a weight basis..

Although there were small differences between the ouabain and digitoxin concentrations in the tissues

**Table 2** The concentrations of [$^3$H]-ouabain and [$^3$H]-digitoxin in the visceral and brain tissue at 60 min after intravenous administration of these drugs

| *Tissue* | *Ouabain (n=6) (pmol/g wet weight) mean ± s.e.* | *Digitoxin (n=3) (pmol/g wet weight) mean ± s.e.* |
|---|---|---|
| Heart | 432 ± 110 | 190 ± 33 |
| Kidney | 3219 ± 1121 | 882 ± 212 |
| Liver | 250 ± 125 | 383 ± 187 |
| Adrenal | 269 ± 147 | 235 ± 133 |
| Skeletal muscle | 56 ± 29 | 73 ± 28 |
| Pituitary | 845 ± 193 | 256 ± 137 |
| Choroid plexus | 204 ± 59 | 670 ± 124 |
| Cerebellum | 11 ± 5 | 26 ± 21 |
| Hypothalamus | 12 ± 6 | 24 ± 17 |
| Pons | 9 ± 6 | 18 ± 13 |
| Medulla | 12 ± 7 | 19 ± 14 |
| Mesencephalon | 10 ± 6 | 21 ± 10 |
| Plasma | 119 ± 20 | 177 ± 68 |
| CSF | 2.9 ± 1.2 | 3.2 ± 3.2 |

**Table 3** The potassium concentration (mEq/ml) of the plasma and cerebrospinal fluid at various times after intravenous administration of saline (control), ouabain, digitoxin, and KCl (1.0 mEq kg$^{-1}$ h$^{-1}$)

| *Time (min)* | | 0 | 5 | 15 | 30 | 45 | 60 |
|---|---|---|---|---|---|---|---|
| Control | Plasma | 3.94 ± 0.16<br>(3)* | 4.03 ± 0.04<br>(3) | 3.83 ± 0.30<br>(3) | 3.95 ± 0.25<br>(3) | 3.97 ± 0.21<br>(3) | 4.02 ± 0.17<br>(3) |
| | CSF | 3.33 ± 0.17<br>(3) | 3.25 ± 0.16<br>(3) | 3.22 ± 0.18<br>(3) | 3.21 ± 0.12<br>(3) | 3.23 ± 0.09<br>(3) | 3.18 ± 0.04<br>(3) |
| Ouabain | Plasma | 4.04 ± 0.62<br>(5) | 4.23 ± 0.78<br>(5) | 4.65 ± 0.79†<br>(5) | 5.10 ± 0.84†<br>(5) | 6.03 ± 1.34†<br>(5) | 5.66 ± 0.84†<br>(4) |
| | CSF | 3.05 ± 0.21<br>(4) | 3.17 ± 0.26<br>(4) | 3.14 ± 0.17<br>(4) | 3.08 ± 0.22<br>(4) | 3.22 ± 0.22<br>(3) | 3.03<br>(2) |
| Digitoxin | Plasma | 3.87 ± 0.51<br>(3) | 3.88 ± 0.66<br>(3) | 3.86 ± 0.62<br>(3) | 3.93 ± 0.66<br>(3) | 3.94 ± 0.88<br>(3) | 4.06 ± 0.64<br>(3) |
| | CSF | 2.72 ± 0.31<br>(3) | 2.82 ± 0.17<br>(3) | 2.82 ± 0.16<br>(3) | 2.76 ± 0.14<br>(3) | 2.80 ± 0.17<br>(3) | 2.81 ± 0.17<br>(3) |
| K$^+$ infusion | Plasma | 4.02 ± 0.17<br>(3) | 4.50 ± 0.14<br>(3) | 4.65 ± 0.45<br>(3) | 4.93 ± 0.49†<br>(3) | 5.02 ± 0.83†<br>(3) | 5.09 ± 0.22†<br>(3) |
| | CSF | 3.18 ± 0.04<br>(3) | 3.31 ± 0.03<br>(3) | 3.32 ± 0.06<br>(3) | 3.20 ± 0.16<br>(3) | 3.38 ± 0.14<br>(3) | 3.30 ± 0.09<br>(3) |

* Number of experiments in parentheses throughout Table.
† Significantly higher than the value at 0 time: $P < 0.05$.

examined, the general pattern of their distribution was similar (Table 2). It is noteworthy that as with ouabain, there was negligible localization of digitoxin in the neural tissue whereas choroid plexus and pituitary concentrated digitoxin to a great extent. Neural tissue concentration of digitoxin was in general less than 15% of the plasma concentrations.

### *Ionic composition of plasma and CSF as affected by arrhythmogenic doses of cardiac glycosides*

The concentrations of potassium, sodium, calcium, and magnesium were measured in plasma and CSF at different times following ouabain and digitoxin infusion. In general, the plasma concentrations of sodium, calcium, and magnesium remained at control levels of 140, 4.0, and 1.0 mEq/litre respectively after the administration of the cardiac glycosides. However, during the 60 min experimental period of ouabain treatment, the plasma [$K^+$] gradually increased from the control level to a level of about 6 mEq/litre while the potassium levels in the CSF remained close to those of the control values (Table 3). The absence of change in CSF [$K^+$] at a time when plasma [$K^+$] was elevated was also seen in a separate series of experiments in which the plasma [$K^+$] was raised by the infusion of 0.9% w/v NaCl solution (saline) containing KCl at a rate of 1.0 mEq $K^+$ $kg^{-1}$ $h^{-1}$ (Table 3). Thus, it appears that neither ouabain nor the potassium infusion altered CSF [$K^+$] concentration. In the experiments with digitoxin, unlike those in which ouabain was used, neither the plasma nor CSF levels of [$K^+$] changed.

## Discussion

The results presented in this study clearly indicate that no specific localization of ouabain or digitoxin occurred in any CNS tissue examined. Furthermore, both glycosides apparently remained within the extracellular space of the brain and did not appear to be generally available for interaction with the neural tissue. Using the terminal plasma level of 118.6 pmol/ml obtained here for ouabain, a substance known to show no significant protein binding (Kuschinsky, 1968), it would be expected that 21 pmol of ouabain would be present in a gram of the tissue due to distribution in the extracellular space, assuming that the extracellular space in brain tissue is approximately 18% (Levin, Fenstermacher & Patlak, 1970). However, all of the brain regions sampled contained substantially smaller amounts of ouabain than the amount that could be accounted for in the extracellular space. Although the lack of accumulation of ouabain by the brain was understandable in terms of physio-chemical limitations of this highly polar compound to penetrate lipid barriers, the low concentration of digitoxin found in the neural tissue was at first unexpected, since digitoxin is exceedingly lipid soluble. However, since as much as 90–95% of the plasma digitoxin may be albumin bound (Kuschinsky, 1968; Lukas, 1971), it might be expected that less than 10 pmol/ml of digitoxin would be available for diffusion from plasma into the brain. On the basis of this amount of free digitoxin present in the plasma, the various brain areas accumulated digitoxin at least 10 times more readily than ouabain, although the absolute tissue: plasma ratios were only slightly higher. No brain region examined showed any selective or unusual digitoxin uptake, although the absence of dysrhythmia with this dose of digitoxin and the possible slower equilibration of digitoxin leaves open the possibility of delayed brain localization of this drug.

The very low CSF/plasma ratio (0.01–0.03) of these cardiac glycosides further indicates that these agents do not easily penetrate the blood brain barrier. Recently, Garan *et al.* (1974) determined the CSF and plasma concentrations of digoxin following intravenous injection. They found that the highest CSF value was 2.3 ng/ml (2.9 pmol/ml) with a CSF/plasma ratio of about 0.05. Although they claimed that the highest value of digoxin in CSF, attained at 15 min, corresponded with the peak coronary vascular effect, in our study the peak value did not correspond to particular physiological correlates. However, they used a bolus injection rather than a loading dose and continuous infusion, so that some differences in time course might be expected.

No specific localization of ouabain occurred in any of the neural areas examined and the mechanism by which ouabain facilitated sympathetic discharge in the hands of Gillis and co-workers (Gillis, 1969; Gillis *et al.*, 1972a & b) remains in doubt. It would be easier to accept a CNS origin of ouabain-induced dysrhythmia if some region of the brain exhibited adequate ouabain-accumulating activity to account for this response. Intracerebral injections of ouabain to produce ventricular dysrhythmia require a cumulative dose of 100 μg (Basu-Ray, Booker, Dutta & Pradhan, 1972; Saxena & Bhargava, 1975), an amount much greater than the amounts of ouabain per g of tissue found in the various dog brain regions in the present experiments. Of course, it is possible that the appropriate brain region was not sampled, although regions previously implicated were included, and it is also possible that a high local concentration in part of the brain escaped detection. Both of these possibilities deserve further study, but for the time being, a CNS site of action for ouabain-induced dysrhythmia is not supported by our results. An alternative, more plausible explanation may implicate peripheral neural structures, including afferents and ganglionic synapses.

Perhaps the site of action of these drugs is in some non-neural structure in the brain such as the choroid plexus or pituitary gland. There is very little information available at present suggesting that the CNS effect of ouabain can be mediated by the pituitary gland. On the other hand, the preferential accumulation of both ouabain and digitoxin in the choroid plexus suggests the possibility that these drugs may influence the active transport of ions and various amines and/or amine metabolites into the CSF, although we could find no change in cation content of CSF.

CSF has relatively more sodium and magnesium and less potassium and calcium than plasma ultrafiltrate and the reported values for the CSF/plasma concentration ratios of sodium, magnesium, potassium, and calcium are 0.95, 1.1, 0.75, and 0.53, respectively (Pollay, 1974). Using an extracorporeal perfusion technique with sheep choroid plexus, Pollay, Kaplan & Nelson (1973) have recently obtained results indicating that the movement of potassium from plasma to CSF is mediated by the Na-K pump which saturates at a plasma concentration of 3.3 mmol/litre and is inhibited by ouabain (10 $\mu$M) in CSF or plasma. The present study shows that ouabain at a free plasma concentration of 0.1 $\mu$M did not greatly alter the $K^+$ concentration in CSF but produced severe arrhythmia.

Although the precise subcellular site of binding for cardiac glycosides in the choroid plexus has yet to be determined, Quinton, Wright & Tormey (1973) have demonstrated by autoradiography that $[^3H]$-ouabain binds to the brush border of the frog ependyma *in vitro*. A recent study on the localization of Na-K ATPase in the choroid plexus of various mammalian species appears to contradict these observations in the dog. Milhorat, Davis & Hammock (1975), using the Ernst technique, found that the ATPase activity is well localized along the basolateral membrane. The contradiction may, of course, only be apparent, for the transport enzyme is not necessarily the sole specific binding site for the drug, and species differences may be important. In the present *in vivo* studies, the drugs may well have bound to the basolateral membrane of choroid plexus initially and subsequently gained access to the brush border after intracellular translocation.

Other workers have recently found preferential accumulation of digoxin in the CSF and choroid plexus of man (Neblett, McNeal, Waltz & Harrison, 1972; Bertler, Anderson & Wettrell, 1973) and dog (DasGupta & Binnion, 1974). These observations made for digoxin compare well with the ouabain and digitoxin accumulation found in the present study. What differences exist for the choroid plexus/plasma concentration ratios, may be understood, in part, in terms of the amount of free drug available in each case. Although it is clear from this study, as well as previous studies (Bertler *et al.*, 1973; DasGupta & Binnion, 1974), that there is a preferential localization of cardic glycoside in the choroid plexus, the pharmacological implication of this binding has yet to be established.

The authors are indebted to Miss Stephanie Brizgys for her assistance during the initial part of this study. The skilful technical assistance of Mr William Marszalec is gratefully acknowledged. This investigation was supported in part by the United States Public Health Services Research Grant HE 07051.

A report of this study has been presented at the Fall Meeting of ASPET, 1975, Davis, California and Indian Pharmacology Society meetings in Madras, India, December 28–30, 1975.

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*Br. J. Pharmac.* (1977), **59**, 107–113

# EFFECT OF *p*-BROMOPHENACYL BROMIDE, AN INHIBITOR OF PHOSPHOLIPASE $A_2$, ON ARACHIDONIC ACID RELEASE AND PROSTAGLANDIN SYNTHESIS BY THE GUINEA-PIG UTERUS *in vitro*

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1 The synthesis of prostaglandins $F_{2\alpha}$ and $E_2$ by guinea-pig uterine homogenates was inhibited by *p*-bromophenacyl bromide (PBPAB), an inhibitor of phospholipase $A_2$.

2 Metabolism of prostaglandin $F_{2\alpha}$ by uterine homogenates was undetectable; this was not affected by PBPAB.

3 There was no significant difference between the amounts of arachidonic acid released from uterine homogenates on days 7 and 15 of the oestrous cycle. Small amounts of dihomo-γ-linolenic acid were detected in the homogenates.

4 The release of arachidonic acid from uterine homogenates was greatly inhibited by PBPAB.

5 Addition of exogenous arachidonic acid to uterine homogenates did not overcome the inhibition of uterine prostaglandin $F_{2\alpha}$ synthesis produced by PBPAB.

6 It is concluded that PBPAB inhibits both the release of arachidonic acid from the guinea-pig uterus and its subsequent conversion into prostaglandins.

## Introduction

Prostaglandin synthesis *in vivo* occurs in several steps. The non-steroidal, anti-inflammatory drugs inhibit the conversion of the precursor fatty acids, such as arachidonic acid, into prostaglandins (Vane, 1971). It is generally recognized that the levels of free arachidonic acid in tissues are too low for any significant production of prostaglandins, and that arachidonic acid is released from some source in order for prostaglandin synthesis to occur. Membrane phospholipids have been regarded for several years as a possible source, the arachidonic acid being released by the action of phospholipase $A_2$ (Lands & Samuelsson, 1968; Vogt, Meyer, Kunze, Lufft & Babilli, 1969; Bartels, Kunze, Vogt & Wille, 1970; Kunze & Vogt, 1971; Flower & Blackwell, 1976). Consequently, inhibition of phospholipase $A_2$ activity should prevent prostaglandin synthesis by tissues.

*p*-Bromophenacyl bromide (PBPAB) is an inhibitor of porcine pancreatic phospholipase (Volwerk, Pieterson & de Haas, 1974). It alkylates the imidazole side-chain of the histidine residue at position 53 of the phospholipase $A_2$ molecule, which contains 120 amino acids in all. A compound which inhibits the release of arachidonic acid from phospholipids, without preventing the conversion of the acid into prostaglandins, would be very useful in studying prostaglandin synthesis by tissues and in elucidating the source of the arachidonic acid. Consequently we have studied the effect of PBPAB on prostaglandin synthesis by, and arachidonic acid release from, the guinea-pig uterus *in vitro*. This tissue is known to synthesize and release prostaglandin $F_{2\alpha}$ under physiological conditions *in vivo* (see Poyser, 1976) and to synthesize predominantly prostaglandin $F_{2\alpha}$ with some prostaglandin $E_2$, from endogenous precursors when homogenized and incubated *in vitro*, (Poyser, 1972).

## Methods

### *Effect of PBPAB on prostaglandin $F_{2\alpha}$ synthesis by the guinea-pig uterus*

Guinea-pigs in dioestrus (vagina closed) were used in groups of four. Prostaglandin synthesizing capacity of the uterus is lower during dioestrus than at the end of the oestrous cycle when the vagina is open (Poyser, 1972). The 4 guinea-pigs in each group were killed by stunning and incising the neck. The uterus from each animal was removed and cut into four equal parts. Quarters from different animals were combined in such a way as to reconstitute one 'uterus' (i.e. upper right horn, upper left horn, lower right horn and lower left horn). This resulted in four uterine samples containing one quarter of the uterus from each animal. Each sample was weighed, and was usually in the range of 0.6 to 0.8 grams. The uterine tissue was opened longitudinally and cut into small pieces. Each sample was placed in 14.5 ml Krebs solution (composition (g/l): NaCl 6.9, KCl 0.354, $CaCl_2$ 0.282, $MGSO_4$, $7H_2O$ 0.294 g, $KH_2PO_4$ 0.162, $NaHCO_3$ 2.1, glucose 2.0), which had been pre-aerated with 5% $CO_2$ in $O_2$. To three of the samples was added PBPAB, dissolved in 0.5 ml ethanol so calculated to give a known final concentration. To the fourth sample was added only 0.5 ml ethanol, and this sample acted as the control. Each sample was incubated in the Krebs solution, aerated with 5% $CO_2$ in $O_2$, at 37°C for 30 minutes. Following this period, the uterine tissue was homogenized in the incubating fluid, using a Fisons' glass homogenizer, and then incubated for another 90 min under the same conditions.

The reaction was stopped by acidifying to pH 4.5 with 1 N HCl and immediately extracting the prostaglandins with ethyl acetate. (The pH of the homogenates following incubation was always in the range of 7.4 to 7.6). The amounts of prostaglandin $F_{2\alpha}$ formed were measured in one of two ways: (1) Initially, the ethyl acetate extracts were further purified, the different prostaglandins separated by column chromatography, and the prostaglandin $F_{2\alpha}$ isolated assayed biologically on the rat fundal strip, by methods described previously, (Poyser, 1972). The identity of the prostaglandin $F_{2\alpha}$ was confirmed by gas chromatography-mass spectrometry. No prostaglandin $F_{1\alpha}$ was detected. (2) The later samples were assayed by radioimmunoassay using an antibody raised and tested in our laboratory (Blatchly & Poyser, 1974; Naylor & Poyser, 1975; Dighe, Emslie, Henderson, Rutherford & Simon, 1975). The double antibody method was used, and two additional cross-reactivity studies were performed. 13,14-Dihydro-15-keto-prostaglandin $F_{2\alpha}$ cross-reacted 0.56% and prostaglandin $D_2$ cross-reacted 17.1%. Previous studies had failed to detect prostaglandin $D_2$ in the uterus, so the relatively high cross-reactivity of this prostaglandin with the $F_{2\alpha}$ antibody was not considered to invalidate the assay.

The ethyl acetate extracts of the incubates were evaporated to dryness and each residue dissolved in 20 ml ethyl acetate. Each solution was then diluted 2 or 4 times with ethyl acetate, and aliquots of 10, 20, 50 and 100 µl were assayed in duplicate. Parallelism (i.e. a similar value obtained for the amount of prostaglandin $F_{2\alpha}$ in the original extract irrespective of volume assayed) was obtained for all samples. This provides good evidence that prostaglandin $F_{2\alpha}$ was being assayed, as substances likely to cross-react do not have standard curves parallel to that of prostaglandin $F_{2\alpha}$. (Prostaglandin $F_{1\alpha}$ is an exception, but the synthesis of prostaglandin $F_{1\alpha}$ by guinea-pig uterine homogenates has not been detected, for reasons apparent later). Consequently, the amount of prostaglandin $F_{2\alpha}$ in each extract was taken as the mean of the 8 values obtained.

Comparable results were obtained by both methods of assay. The amount of prostaglandin $F_{2\alpha}$ synthesized by 100 mg of uterine tissue was calculated for each sample. The percentage inhibition of prostaglandin $F_{2\alpha}$ synthesis produced by PBPAB was established for each group of four guinea-pigs.

### *Effect of PBPAB on the metabolism of prostaglandin $F_{2\alpha}$ by the guinea-pig uterus*

The uterus of a guinea-pig in dioestrus was removed and each uterine horn divided in half. The top half of one horn was combined with the bottom half of the other horn, and each uterine sample was weighed. The uterine tissue was opened longitudinally and cut into small pieces. One sample was placed in 14.5 ml Krebs solution to which was added PBPAB, dissolved in 0.3 ml ethanol, to give a final concentration of 100 µg/ml. The other sample was placed in 14.5 ml Krebs solution to which was added 0.3 ml ethanol. Both samples were incubated at 37°C for 30 min, aerated with 5% $CO_2$ in $O_2$, then homogenized in the incubation fluid. To each homogenate was added 0.4 µCi [9-$^3$H]-prostaglandin $F_{2\alpha}$ (sp.act. 7.5 Ci/mmol, New England Nuclear) dissolved in 0.2 ml methanol. The homogenates were incubated for another 90 min, and the prostaglandins and any metabolites formed extracted with ethyl acetate. Each extract was then further purified by dissolving in 20 ml 67% ethanol and partitioning twice with 20 ml petroleum ether (b.p. 60°–80°C). The ethanol fraction was evaporated to dryness. The residue was dissolved in a mixture of 0.1 ml methanol and 0.1 ml toluene and spotted on to a thin-layer chromatography plate of silica gel (20 cm x 5 cm; Merck). The plate was developed in two solvent systems: the FVI system of Andersen (1969) and the GCM system of Millar (1974). The plates were scanned with a Panax thin-layer scanner RTLS-1A to locate the radioactive spots. A plate containing authentic [9-$^3$H]-prostaglandin $F_{2\alpha}$ was also run. This experiment was performed on two guinea-pig uteri.

### *Effect of PBPAB on prostaglandin $E_2$ synthesis by the guinea-pig uterus*

The effect of PBPAB on uterine prostaglandin $E_2$ synthesis was only examined in the later stages of this study when a reliable and sensitive method of measuring low levels of prostaglandin $E_2$ became available. An antibody to prostaglandin $E_2$ was purchased from the Pasteur Institute, Paris, where the antibody had been raised and tested (Dray, Charbonnel & Maclouf, 1975). In our double-antibody method, the antibody had the cross-reactivities listed in Table 1. The working range of the assay was from 3 to 80 pg prostaglandin $E_2$. The coefficient of variation for within assays (the intra-assay precision) was 13.3% calculated from 354 samples (of guinea-pig, sheep and human uterine extracts) in 12 assays. The inter-assay precision was calculated from the results obtained by assaying in duplicate at least two different aliquots of a standard solution containing 1 ng/ml prostaglandin $E_2$. The coefficient of variation between assays was 9.9%.

Accuracy was assessed in two ways. Firstly, the mean ($\pm$ s.e. mean) of the values obtained from the 12 assays for the standard solution of 1 ng/ml prostaglandin $E_2$ was $0.96 \pm 0.2$ ng/ml. Secondly, a guinea-pig uterus was homogenized and incubated as described previously (Poyser, 1972). Prostaglandins were extracted and the amount of prostaglandin $E_2$ formed was assayed before and after silicic acid column chromatography. A control column, on which 20 ng prostaglandin $E_2$ was placed, was run in parallel to correct for procedural losses incurred during the chromatography step. The values obtained for the amount of prostaglandin $E_2$ formed were 29.3 ng when assayed before chromatography and 29.2 ng (corrected for a recovery of 61.5% from the control column) when assayed after chromatography. The amount of prostaglandin $E_2$ produced by 100 mg tissue was, therefore, 4.9 ng when assayed by both techniques. This value is in the expected range (Poyser, 1972).

Consequently, the antibody could measure prostaglandin $E_2$ accurately, and with sufficient precision, using ethyl acetate extracts of guinea-pig uterine homogenates. The amounts of prostaglandin $E_2$ synthesized by the guinea-pig uterine homogenates in the absence and presence of PBPAB (100 μg/ml) were determined in a similar manner to that of prostaglandin $F_{2\alpha}$ using radioimmunoassay. Parallelism was again achieved providing further evidence that prostaglandin $E_2$ was being assayed in each sample.

**Table 1** Cross-reactivities of several prostaglandins and prostaglandin metabolites with the prostaglandin $E_2$ antibody

| *Prostaglandin* | *% Cross-reactivity* |
|---|---|
| $PGE_1$ | 15.1 |
| $PGF_{2\alpha}$ | 0.17 |
| $PGA_2$ | 0.14 |
| $PGB_2$ | 0.05 |
| 15 Keto-$PGE_2$ | 0.58 |
| 13,14 Dihydro-15 keto-$PGE_2$ | 0.06 |

### *Release of arachidonic acid from the guinea-pig uterus at times of low and high prostaglandin synthesis*

Five guinea-pigs were killed on day 7 of the oestrous cycle (low prostaglandin synthesis) and day 15 (high prostaglandin synthesis) and their uteri removed. Each uterus was divided into four equal parts, and the top half of one uterine horn was combined with the bottom half of the opposite horn. The two uterine samples were weighed. One sample was then immediately homogenized in 15 ml ice-cold Tyrode solution (composition (g/l): NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, $NaH_2PO_4$ 0.05, $NaHCO_3$ 1.0, glucose 1.0), which contained indomethacin (50 μg/ml). To the homogenate was added 0.02 μCi [$^{14}$C]-arachidonic acid (sp.act. 58 mCi/mmol; Amersham) and the lipids extracted as described below. The second sample was homogenized in 15 ml Tyrode solution containing indomethacin and the homogenate incubated for 60 min at 37°C, aerated with $O_2$. [$^{14}$C]-arachidonic acid (0.02 μCi) was then added, the pH lowered to 4.5 with 1 N HCl and the lipids extracted by partitioning three times with 30 ml ethyl acetate. The ethyl acetate fractions were combined and evaporated to dryness. The residue was dissolved in 20 ml 67% ethanol and the fatty acids extracted by partitioning three times with 20 ml petroleum ether (b.p. 60°–80°C). The petroleum fractions were combined and evaporated to dryness. Arachidonic acid was separated from other fatty acids in the extract by reversed-phase partition chromatography using lipidex 5000 (Becker-Delft, Holland) with a solvent system of methanol, water, 1,2-dichloroethane and glacial acetic acid in the ratio of 800:200:100:1.1 (G. Jones, personal communication). The $^{14}$C-label enabled the tracing of arachidonic acid on the column, and a correction to be made for procedural losses following isolation (average recovery $= 67.6 \pm 1.3\%$, $n = 33$). The samples of arachidonic acid isolated were assayed by gas chromatography using a Pye series 104 chromatograph containing a column of 4% OV1 on supelcoport or 5% SP 2330 on chromasorb W. 2,3-Benzofluoroanthene was employed as an internal standard. In several instances the identity of the arachidonic acid was confirmed by gas chromatography-mass spectrometry. The amounts of free arachidonic acid released per 100 mg tissue before and after incubation were then calculated.

Two further guinea-pigs were examined for the presence of dihomo-γ-linolenic acid in the uterus in a similar manner, using [$^{14}$C]-dihomo-γ-linolenic acid as the tracer.

### *Effect of PBPAB on arachidonic acid release from the uterus*

Five guinea-pigs were killed on day 7 of the oestrous cycle and the uterus removed. Each uterus was divided into six equal parts, and one-third of one uterine horn combined with one-third of the opposite horn obtained from a different region. The three uterine samples were weighed. One sample was immediately homogenized in ice-cold Krebs solution containing indomethacin (50 µg/ml) and the arachidonic acid content measured as just described. The uterine tissue in the other two samples was opened longitudinally, then cut into small pieces. One sample was placed in Krebs solution containing indomethacin (50 µg/ml), whilst the other sample was placed in Krebs solution containing indomethacin (50 µg/ml) and PBPAB (100 µg/ml) (all samples contained 0.6 ml ethanol, used for dissolving the compounds). These samples were incubated for 30 min at 37°C, aerated with 5% $CO_2$ in $O_2$. Both samples were then homogenized and incubated under the same conditions for a further 60 minutes. Arachidonic acid content was then estimated as before.

### *Effect of exogenous arachidonic acid and PBPAB on prostaglandin $F_{2\alpha}$ synthesis by the uterus*

Five groups of 4 guinea-pigs were used. The guinea-pigs were in dioestrus. The uteri from the animals in each group were removed and divided into four equal parts. One quarter of each uterus was combined so as to reconstitute one 'whole uterus' in the manner previously described. The four uterine samples were weighed, and the uterine tissue opened longitudinally and cut into small pieces. One sample from each group was incubated for 30 min at 37°C, in 15 ml of one of the following solutions: (1) Krebs solution (control) (2) Krebs solution containing 100 µg/ml PBPAB. (3) Krebs solution containing 10 µg/ml arachidonic acid. (4) Krebs solution containing 100 µg/ml PBPAB and 10 µg/ml arachidonic acid.

All samples contained an equal amount of ethanol (0.6 ml). The arachidonic acid had been previously purified by reversed-phase partition chromatography by Dr G. Jones.

After the 30 min incubation, each sample was homogenized in its incubation fluid and further incubated under the same conditions for 90 minutes. The amounts of prostaglandin $F_{2\alpha}$ synthesized were determined by radioimmunoassay.

## Results

### *Effect of PBPAB on prostaglandin $F_{2\alpha}$ synthesis by the guinea-pig uterus*

Prostaglandin $F_{2\alpha}$ synthesis by homogenates of guinea-pig uteri was inhibited by PBPAB (Figure 1). Increasing the concentration by PBPAB increased the inhibition of synthesis, although two plateaux of inhibition were seen. The maximum inhibition of synthesis obtained was 70 to 80%. The concentration of PBPAB, could not be increased because of its limited solubility.

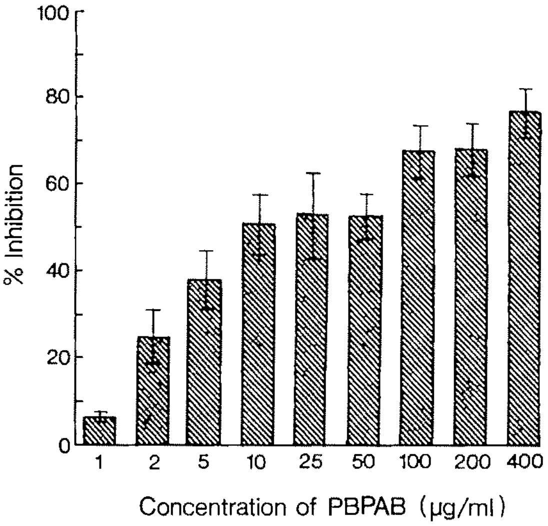

**Figure 1** Effect of *p*-bromophenacyl bromide (PBPAB) on prostaglandin $F_{2\alpha}$ synthesis by homogenates of guinea-pig uteri.

### *Effect of PBPAB on metabolism of prostaglandin $F_{2\alpha}$ by the guinea-pig uterus*

Previous studies have shown that, under the conditions used, there is no detectable metabolism of prostaglandin $F_{2\alpha}$ by the guinea-pig uterus, even at different stages of the cycle (Maule Walker, 1975). This study confirmed that observation in so far as all the thin layer chromatography plates had only one spot containing radioactivity at $R_F$ values between 0.43 and 0.45. The $R_F$ value of [9-$^3$H]-prostaglandin $F_{2\alpha}$ was 0.43. PBPAB does not, therefore, affect metabolism of prostaglandin $F_{2\alpha}$ by the uterus, and metabolism remained undetectable.

### *Effect of PBPAB on prostaglandin $E_2$ synthesis by the uterus*

At the one concentration of PBPAB studied (100 µg/ml), protaglandin $E_2$ synthesis by guinea-pig uterine homogenates was inhibited by 63.0 ± 4.3% (mean ± s.e., $n = 5$). The absolute amounts of prostaglandin $E_2$ synthesized were considerably less than the amounts of $F_{2\alpha}$ synthesized, confirming earlier studies (Poyser, 1972; Maule Walker & Poyser, 1974).

### *Release of arachidonic acid from the guinea-pig uterus at times of low and high prostaglandin synthesis*

The results are shown in Table 2. The incubation of guinea-pig uterine homogenates for 60 min significantly increased ($P < 0.01$) the levels of free arachidonic acid detectable on both day 7 and day 15. There was no significant difference between the levels on day 7 (low prostaglandin synthesizing capacity) and day 15 (high prostaglandin synthesizing capacity) when compared before or after incubation.

In the two additional guinea-pig uteri studied, the presence of dihomo-$\gamma$-linolenic acid following incubation was just detectable, but was not measurable. There was about 1% or even less of the amount of arachidonic acid present.

### *Effect of PBPAB on arachidonic acid release from the uterus*

The results are shown in Table 3. The pre- and post-incubation (without PBPAB) levels of arachidonic acid are not significantly different from those in Table 2. The addition of PBPAB (100 µg/ml) to the homogenate, however, reduced the amount of arachidonic acid released following incubation by 75.9%.

**Table 2** Amounts of arachidonic acid (mean ± s.e.) in guinea-pig uterine homogenates before and after incubation ($n = 5$)

| | *Amount of arachidonic acid (µg/100 mg tissue)* | |
|---|---|---|
| | *Day 7* | *Day 15* |
| Before incubation | $2.1 \pm 0.4^{a}$ | $1.7 \pm 0.1^{a}$ |
| After incubation | $5.1 \pm 0.4^{b}$ | $5.0 \pm 1.0^{b}$ |

Values with the same superscript are not significantly different.

**Table 3** Effect of *p*-bromophenacyl bromide (PBPAB, 100 µg/ml) on arachidonic acid release from guinea-pig uterine homogenates ($n = 5$).

| | *Amount of arachidonic acid (µg/100 mg tissue) (mean ± s.e.)* |
|---|---|
| Non-incubated | $1.6 \pm 0.4^{a}$ |
| Incubated | $4.5 \pm 0.4^{b}$ |
| Incubated with PBPAB | $2.3 \pm 0.4^{a}$ |

Values with the same superscript are not significantly different.

### *Effect of exogenous arachidonic acid and PBPAB on prostaglandin $F_{2\alpha}$ synthesis by the uterus*

This experiment was designed to investigate whether adding exogenous arachidonic acid could overcome the inhibition of uterine prostaglandin $F_{2\alpha}$ synthesis produced by PBPAB. The results are shown in Table 4. It is apparent that adding arachidonic acid to the incubate did not overcome the inhibitory action of PBPAB. Furthermore, the addition of exogenous arachidonic acid did not increase uterine prostaglandin $F_{2\alpha}$ synthesis, in samples without PBPAB, above control levels. This last observation confirms earlier studies (Poyser, 1971).

## Discussion

A compound which inhibits phospholipase $A_2$ without preventing the conversion of precursor fatty acids into prostaglandins would be very useful in the overall study of prostaglandin synthesis, especially in establishing the source of the arachidonic acid. This study has shown that PBPAB, an inhibitor of porcine pancreatic phospholipase $A_2$ (Volwerk *et al.*, 1974), inhibits the synthesis of prostaglandin $F_{2\alpha}$ from endogenous precursors by the guinea-pig uterus, when homogenized and incubated *in vitro*. The results obtained are not due to a redirection of synthesis towards prostaglandin $E_2$ as its synthesis was also inhibited by PBPAB. Also, the results are not due to an increase in metabolism induced by PBPAB, as metabolism remained undetectable. These results do not show whether the inhibition of prostaglandin synthesis was due to a lack of arachidonic acid in the incubate or to an inhibition of its conversion into prostaglandins.

Consequently, the effect of PBPAB on arachidonic acid release from the guinea-pig uterus was also examined. It was found that this compound did reduce quite markedly the increase in level of free arachidonic acid which occurs during the incubation of uterine

**Table 4** Effect of exogenous arachidonic acid (10 µg/ml) on the inhibition of uterine prostaglandin $F_{2\alpha}$ synthesis produced by *p*-bromophenacyl bromide (PBPAB, 100 µg/ml) ($n = 5$).

| *Treatment* | *% Inhibition (mean + s.e.)* |
|---|---|
| Control homogenates | — |
| Homogenates + arachidonic acid | 6.2 + 4.6 |
| Homogenates + PBPAB | 74.0 + 4.1* |
| Homogenates + PBPAB and arachidonic acid | 63.6 + 5.8* |

* not significantly different.

homogenates. Whether this is due to it solely inhibiting phospholipase $A_2$ has still to be established. There are other sources of arachidonic acid besides phospholipids, such as triglycerides and cholesterol esters. Consequently, an inhibition of triglyceride lipases and cholesterol esterase may also contribute to the lowering in the level of free arachidonic acid.

It is apparent that the level of arachidonic acid remaining after incubation of the uterus with PBPAB is still far in excess of the amounts (usually 40 to 150 ng/100 mg tissue) of prostaglandin $F_{2\alpha}$ synthesized. Furthermore, the addition of exogenous arachidonic acid did not overcome the inhibition of prostaglandin $F_{2\alpha}$ synthesis produced by PBPAB. This compound therefore inhibits both the release of arachidonic acid from uterine tissue and the conversion of arachidonic acid into prostaglandins. PBPAB is only suitable for determining the source of arachidonic acid if one measures the levels of this acid and does not rely on prostaglandin synthesis as the end-point.

Several drugs have been investigated for their ability to inhibit phospholipase $A_2$. Local anaesthetics and tranquillisers prevent the release of arachidonic acid from phospholipids, but they also, with the exception of amethacine, inhibit the conversion of arachidonic acid into prostaglandins (Seppälä, Saris & Gauffin, 1971; Scherphof, Scarpa & van Toorenenbergen, 1972; Kunze, Bohn & Vogt, 1974; Kunze, Bohn & Bahrke, 1975). Propranolol also prevents the release of arachidonic acid from phospholipids (Seppälä *et al.*, 1971). However, these drugs do not inhibit phospholipase $A_2$ directly like PBPAB. Earlier evidence suggested they interacted with $Ca^{2+}$ (Scherphof *et al.*, 1972; Waite & Sisson, 1972) although later evidence suggests they interact with the phospholipids (Scherphof & Estenberg, 1975). Vane and his co-workers have used mepacrine as a phospholipase A inhibitor (Vane, 1976), though this drug, like other antimalarials, is a good inhibitor of triglyceride lipases (Markus & Ball, 1969). It also inhibits prostaglandin synthetase (Flower & Blackwell, 1976) so mepacrine would also appear unsuitable to use as a specific phospholipase $A_2$ inhibitor. Perhaps amethacine should be investigated further.

An interesting finding in the present study concerns arachidonic acid release from uterine homogenates following incubation. There was no difference in the amounts released between day 7 of the oestrous cycle (low prostaglandin synthesis) and day 15 (high prostaglandin synthesis). Consequently, on day 7 there is about 1% conversion of the available arachidonic acid into prostaglandins, which increases to about 2 to 3% by day 15. It follows that limitation of prostaglandin synthesis on day 7 is not due to lack of precursor. It is therefore not surprising that the addition of exogenous arachidonic to day 7 uterine homogenates did not increase prostaglandin $F_{2\alpha}$ synthesis. The increase in synthesis on day 15 is due to an increase in the amount of prostaglandin synthetase in the uterus. Its level is apparently controlled by oestradiol, as the treatment of ovariectomized guinea-pigs with oestradiol increases the prostaglandin $F_{2\alpha}$ synthesizing capacity of the uterus (Naylor & Poyser, 1975). Oestradiol output from the ovary does increase towards the end of the oestrous cycle (Joshi, Watson & Labhsetwar, 1973). Similar findings have been reported for the rat uterus concerning the control of synthetase levels (Ham, Cirillo, Zanetti & Kuehl, 1975).

The precursor of the l-series prostaglandins (e.g. prostaglandins $F_{1\alpha}$ and $E_1$) dihomo-$\gamma$-linolenic acid, was just detectable in uterine homogenates following incubation. There was probably 100 times more of the arachidonic acid present. Consequently, it is not surprising that neither prostaglandin $F_{1\alpha}$ nor $E_1$ have been detected in extracts of guinea-pig uterine homogenates.

The levels of free arachidonic acid following homogenization of the uterus but before incubation were remarkably high. It has been reported for the rat brain that there is a rapid release of arachidonic acid during the 10 min period following death (Wolfe, Pappius & Marion, 1976). This may have occurred in the uterus especially as the time of death to the extraction of arachidonic acid was 10 to 15 minutes. Consequently improved techniques of stopping enzyme action at the time of death will have to be used to establish the true levels of free arachidonic acid. Furthermore, it may prove difficult to establish from where the free 1 to 3% of the total arachidonic acid is derived for prostaglandin synthesis even with the use of specific enzyme inhibitors, if indeed there is a specific source.

Another problem which may be difficult to solve is the actual place where arachidonic acid release and prostaglandin synthesis takes place in the intact uterus, *in vivo*. Both may occur in the endometrium or in the myometrium, or there may even be an interaction between the two sites. Prostaglandin synthesis is likely to take place in the endometrium since its chemical destruction prolongs the oestrous cycle in the guinea-pig (Butcher, Chu & Melampey, 1962). So far, it has proved extremely difficult to separate the guinea-pig uterus into the endometrium and myometrium *in vitro*, so as to resolve this point.

This work was supported by an M.R.C. grant to Professor E.W. Horton, whose encouragement in this study was much appreciated. We appreciate the technical work of Mrs L. Simon and Miss A. Pelanis in performing the radioimmunoassays. Authentic prostaglandins were provided by the Upjohn Company and indomethacin by Merck, Sharp and Dohme Ltd. Information regarding phospholipase $A_2$ inhibitors was kindly provided by Professor Dr G.H. de Haas, Biochemical Laboratories, Utrecht, The Netherlands.

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(Received May 11, 1976.
Revised June 8, 1976.)

*Br. J. Pharmac.* (1977), **59**, 115–121

# EFFECT OF HARMALINE ON SODIUM TRANSPORT IN *Rana esculenta* SKIN

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1 Harmaline, together with certain hallucinogenic alkaloids of the same group (harmine, 2 methyl harmine) stimulates sodium transport across the *in vitro* skin of *Rana esculenta* when it is added to the external medium at a low concentration (0.1 mM). This effect is due to an increase of the sodium influx, and is reversed by washing. It is suggested that harmaline intervenes at the sodium penetration sites at the external face of the transport compartment.

2 At a higher concentration (5 mM) added to the internal medium harmaline inhibits sodium net absorption. The inhibition is due mainly to an increase of the efflux, while the influx may be either inhibited or increased. Under these conditions the influx becomes insensitive to amiloride. It is suggested that the inhibition of sodium transport is the result of harmaline interfering with a transport ATPase, and also that harmaline induces new sites for the passage of sodium.

## Introduction

Harmaline, like certain compounds of the indolealkaloid group is a hallucinogen (Hoffer & Osmond, 1967; Schultes, 1969). According to Canessa, Jaimovich & de la Fuente (1973) these substances act as Na/K ATPase inhibitors at the $Na^+$-dependent phosphorylation level. They noted an inhibitory effect of harmaline in ATPase preparations of human red blood cells, rat brain and squid retinal axon and also showed that harmaline reduced sodium transport across the squid axon and in frog skin. Sepulveda & Robinson (1974) reported inhibition of sodium transport in mammalian intestinal mucosa and kidney cells by harmaline and suggested that harmaline interacts with the sodium binding sites of the transport carriers.

The experiments on the isolated skin of *Rana esculenta* recorded here, show that harmaline modifies the sodium transport of this epithelium. Added to the epithelial face of the preparation it stimulates sodium transport while adition to the serosal face results in a diminution of the short circuit current. It is proposed that the activating effect of harmaline is an action on the permeability of most external barriers while the inhibitory effect is probably due to interference between harmaline and a transport ATPase.

Preliminary accounts of some of these observations have been published (Ehrenfeld & Garcia-Romeu, 1975).

## Methods

The experiments were carried out on isolated abdominal skins of *Rana esculenta.* The animals were kept, unfed, in running tap water at 15°C. The skins were mounted in lucite double chambers, the exposed areas being 7 $cm^2$ or 1.54 $cm^2$ according to the type of chamber used. Each side of the skin was bathed with either 7 ml or 3 ml of Ringer, circulated by an airstream. The potential difference (PD) between epithelial and corial bathing solutions was measured with calomel electrodes and agar-KCl bridges. For measurement of the short circuit current (SCC) the counter potential was applied with Ag–AgCl electrodes coupled to an automatic SCC apparatus. All experiments were preceded by an equilibration period of an hour.

For the measurements of $Na^+$ influx, $^{22}Na$ (20 µCi/100 ml) was added to the epithelial bathing solution and after 20 min for equilibration, its appearance in the internal chamber was followed with time. The radioactivity of the samples was measured in a Mecaserto well counter MO 13/100. Influxes were calculated from the quantity of radioisotope transferred per unit time and the specific radioactivity in the external compartment.

When the mucous face of the skin was bathed with dilute NaCl solution (2 mM) the sodium transport was studied in open circuit conditions and sodium concentration was measured by flame photometry with an

Eppendorf photometer. The net fluxes of $Na^+$ were calculated from the slopes of the sodium concentrations in the external medium as a function of time. In the 2 mM/Ringer experiments the sodium efflux was obtained as the difference between influx, measured with $^{22}Na$, and netflux. All fluxes are expressed in nEq $h^{-1}$ $cm^{-2}$.

Ringer solution of the following composition (mM) was used; NaCl 111, $NaHCO_3$ 2.4, KCl 2.0, $CaCl_2$ 1.0 and glucose 11.1.

Dilute sodium chloride medium contained 2 mM NaCl and 2 mM imidazole adjusted to pH 7.2 with $H_2SO_4$; amiloride (Merck, Sharp & Dohme Research Laboratories, West Point, Pa) was added to the external medium; ouabain (Calbiochem) was added to the internal medium; harmaline, harmine and 2 methyl harmine (Sigma Chemical Co., St. Louis, Missouri) were added either to the external or internal medium.

## Results

### *Stimulation by harmaline added to the external medium of transepithelial sodium transport*

Harmaline (0.2 mM) added to the epithelial face of the skin produced an immediate increase of the SCC (Figure 1a) and of the transepithelial PD and also a slight reduction of the resistance (Table 1). Table 2 A shows that this SCC increase is due to an increase of the sodium influx. The SCC increase was immediate but the time for the maximal response was variable: frequently it was approximately 1 min but varied from 10–20 s to 20 minutes. In the continued presence of harmaline the SCC values were relatively stable with a tendency to fall with time. The SCC and the PD returned to their normal values when preparations were washed.

When added to the external medium at a concentration of 0.2 mM harmine and 2 methyl harmine have effects similar to those of harmaline on the SCC; the stimulation of the SCC induced by these three chemically related compounds was respectively 57%, 60% and 44%. The stimulatory effect was in all instances reversible.

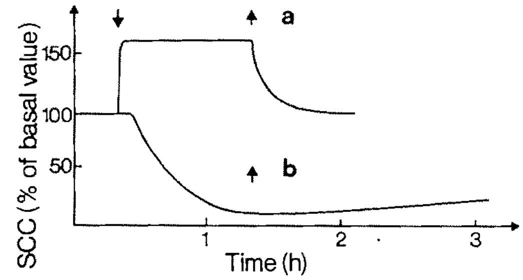

**Figure 1** Effect of harmaline on short circuit current (SCC) added to the external (a) or internal (b) medium. Concentrations: external medium 0.2 mM, internal 5 mM. Addition of hallucinogen and washing marked by arrows. Ordinate scale: variation of SCC as per cent of basal value; abscissa scale: time in hours. The values of SCC in experiment (a) were: control 1803 nEq $h^{-1}$ $cm^{-2}$, in the presence of harmaline 2884 nEq $h^{-1}$ $cm^{-2}$ and after washing 1790 nEq $h^{-1}$ $cm^{-2}$. In experiment (b) control 1205 nEq $h^{-1}$ $cm^{-2}$, in the presence of harmaline (maximal effect) 125 nEq $h^{-1}$ $cm^{-2}$ and after washing 241 nEq $h^{-1}$ $cm^{-2}$.

When the skin was mounted in open circuit with the epithelial face bathed with dilute NaCl, addition of harmaline (0.1 mM) to this solution increased the sodium netflux due to an augmentation of the influx (Table 2B).

### *Concentration-response curve: harmaline added to external medium*

Figure 2 shows that the threshold of the stimulatory effect of harmaline was between 1 and 10 µM and its maximal effect was at 1 mM. Higher concentrations

**Table 1** Variation of the electrical parameters of the frog skin after addition of harmaline

| | *PD* | *SCC* | *R* |
|---|---|---|---|
| Control (*n*=7) | 86.3±4.6 | 1790±153 | 1879±211 |
| Harmaline 0.2 mM (*n*=7) | 110.0±6.4 | 2631±201 | 1625±185 |
| Diff. | +23.7±4.5** | +841±130** | −254±95* |

Short circuit current (SCC), potential difference (PD) and transepithelial resistance (*R*) in nEq $h^{-1}$ $cm^{-2}$, mV and $\Omega/cm^2$ respectively before and after the addition of harmaline (0.2 mM) to the external medium. Number of skins in parentheses. Diff.: Difference of paired data ±s.e. mean.
* $P < 0.05$; ** $P < 0.001$.

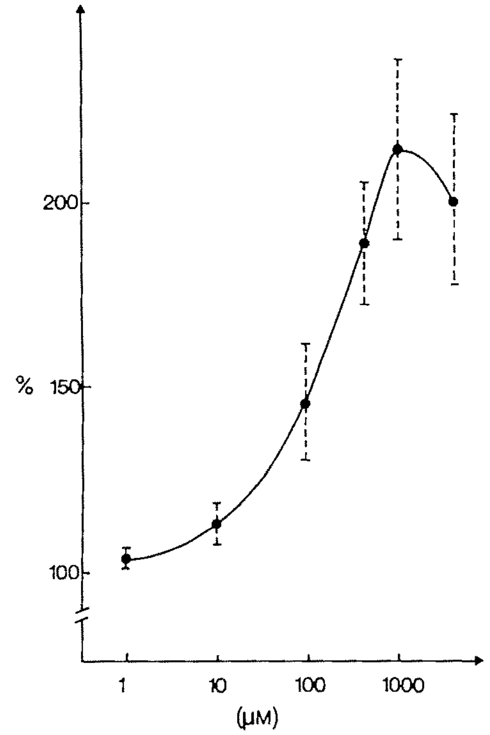

**Figure 2** Concentration-response curve to harmaline added to external face of skins. Ordinate scale: short circuit current (SCC) as per cent increase relative to basal value; abscissa scale: logarithm of hallucinogen concentration. Number of experiments $n=5$. Mean value of initial current 728 ± 177.

(5 mM) stimulated the SCC less than 1 mM. At these concentrations current stimulation was sometimes followed by a slight inhibition, giving a biphasic response.

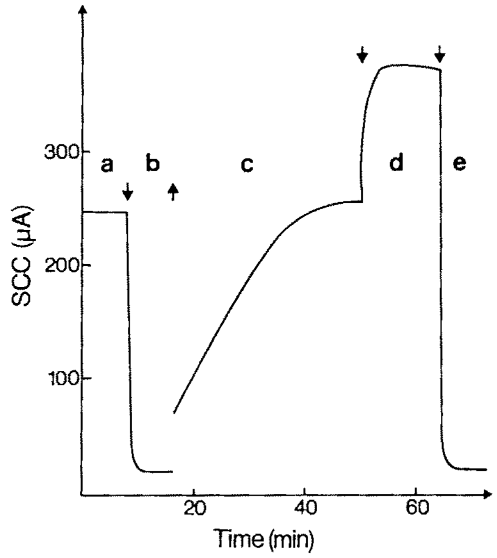

**Figure 3** Short circuit current (SCC) of a skin during 5 successive periods; (a) control, (b) addition of amiloride (10 μM) to external medium, (c) washing, (d) addition of harmaline (0.2 mM) to the external medium, (e) addition of amiloride (10 μM) in presence of harmaline. Ordinate scale: SCC in μA; abscissa scale: time in minutes. Area 7 cm$^2$.

### *Amiloride-harmaline interactions*

To investigate interactions between amiloride (an inhibitor of Na ion translocation at the external face) and harmaline, inhibition by amiloride was studied in the presence and absence of harmaline. Amiloride was used at a concentration of 10 μM and harmaline added to the external medium at a concentration of 0.2 mM. One such experiment is shown in Figure 3. After a control period (a) amiloride added in the external

**Table 2** The effect of harmaline applied to the external solution on transepithelial sodium transport

| | | $J_{in}$ $Na^+$ | SCC | |
|---|---|---|---|---|
| A | Control | 1008 ± 184 | 1080 ± 224 | |
| | Harmaline | 1222 ± 194 | 1395 ± 198 | |
| ($n=8$) | Diff. | 214 ± 55*** | 315 ± 97** | |
| | | $J_{in}$ $Na^+$ | $J_{net}$ $Na^+$ | $J_{out}$ $Na^+$ |
| | Control | 140 ± 27 | +71 ± 26 | 69 ± 19 |
| B | Harmaline | 193 ± 33 | +105 ± 32 | 88 ± 24 |
| ($n=5$) | Diff. | 53 ± 10*** | 34 ± 10* | 19 ± 7* |

Two experimental conditions: (A) short circuited skins bathed in Ringer solutions, (B) open-circuit skins with external face bathed with dilute (2 mM) NaCl solution. $J_{in}$, $J_{out}$, SCC, $J_{net}$=influx, efflux, short circuit current and net flux respectively. All values in nEq h$^{-1}$ cm$^{-2}$ ± s.e. mean. In expts A harmaline added in concentration of 0.2 mM; in B 0.1 mM. Number of skins in parentheses. Diff: Difference of paired data ± s.e. mean.
*$P<0.05$; **$P<0.02$; ***$P<0.01$.

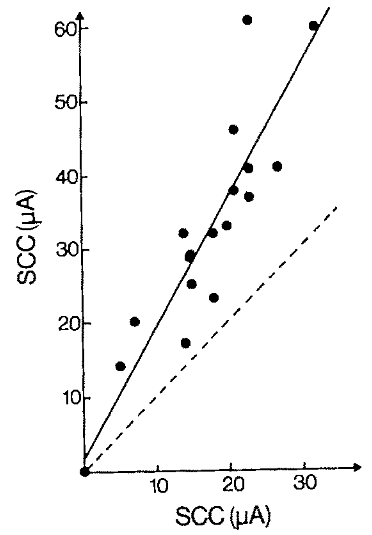

**Figure 4** Stimulation of short circuit current (SCC) by harmaline (0.1 mM) added to external medium after various degrees of inhibition of Na transport by ouabain. Ordinate scale: maximal response of SCC after addition of harmaline to external medium; abscissa scale: SCC remaining after ouabain treatment. SCC expressed in μA. Area: 7 cm$^2$. Equation: SCC (ouabain+harmaline)=1.77 SCC (ouabain)+1.36 ($r$=0.93).

medium induced a strong inhibition of sodium transport (b) and washing restored the SCC to a value similar to the control period (c). Addition of harmaline induced a stimulation of the SCC (d) and amiloride (e) in the presence of harmaline inhibited the sodium transport to the same extent as in period (b). The means and standard errors of transport rates (in nEq h$^{-1}$ cm$^{-2}$) on a group of 4 skins were as follows: control 1535±273; amiloride in the external medium 183±48; washing 1500±371; addition of harmaline to the external medium 2031±448; addition of amiloride in presence of harmaline 170±38. The current level after amiloride inhibition was the same irrespective of the presence of harmaline. Furthermore the addition of harmaline (0.2 mM) in the presence of amiloride (1 μM) had no effect on SCC.

### *The effect of external harmaline on ouabain-pretreated skins*

The stimulatory effects of harmaline (0.1 mM) were studied on skins of *R. esculenta* previously treated with ouabain in concentrations varying from 5 μM to 5 mM. The results from 4 groups of 5 animals are represented in Figure 4. The increase of SCC brought about by the addition of harmaline was found to be proportional to the SCC value remaining after inhibition by ouabain. When the inhibition was total, harmaline could not restore Na transport.

### *Action of harmaline added to the internal medium*

Table 3 summarizes the effect on the SCC of the addition of harmaline at two different concentrations to the internal medium. At 0.1 mM, after a long latent period, it produced a slight transitory increase of the SCC with a maximum at about 20 minutes. At a dose 50 times higher (5 mM) it reduced the SCC (Figure 1b, Table 3); this inhibition started after a few minutes, was about 30% after 30 min and 85% after 1.5 hours. It was not readily reversed by washing.

In a different type of experiment, unidirectional Na$^+$ fluxes and the SCC were followed after the addition of harmaline to the internal medium (Table 4). The experiments covered 3 periods of 45 min: a control period, one in which harmaline (5 mM) was added to the internal solution and one with both external amiloride (50 μM) and harmaline in the internal medium. Following the control period, measurements were started 90 min after the addition of the harmaline. The results of 25 skins tested in this way

**Table 3** The effect of harmaline (5 mM or 0.1 mM) added to the internal medium

| *Dose* | *Control t=0* | *t=30 min* | *t=60 min* | *t=90 min* |
|---|---|---|---|---|
| 5 mM | 1085±182<br>($n$=10) | 759±165<br>($n$=10) | 384±85<br>($n$=10) | 165±87<br>($n$=9) |
| % inhibition | | 30% | 65% | 85% |
| | *t=0* | *t=20 min* | *t=44 min* | |
| 0.1 mM | 812±125<br>($n$=8) | 919±141<br>($n$=8) | 788±146<br>($n$=8) | |

After a control period, harmaline was added to the internal medium and the SCC followed as a function of time. Current in nEq h$^{-1}$ cm$^{-2}$. Number of skins in parentheses. Decrease of the SCC in per cent.

fell into two groups: group A in which harmaline reduced the sodium influx and group B in which it did not. In both groups the addition of harmaline to the internal medium made the epithelium incapable of carrying out a net sodium transport since the effluxes were increased to the same level as the influxes. In these conditions the influx of Na was no longer sensitive to amiloride.

## Discussion

Harmaline added at 10 μM to 0.5 mM to the mucosal face of the skin increases the transepithelial transport of sodium. This stimulation of $Na^+$ net transport is due to an increase of $Na^+$ influx and was found to occur under two experimental conditions: when the skin is short-circuited and bathed on both sides by Ringer, and when it is in open circuit conditions with a dilute sodium chloride solution at the external face. An accepted model of the penetration of sodium is that there are two barriers limiting the transport compartment: an external one permeable to sodium and impermeable to potassium (Koefoed-Johnsen & Ussing, 1958) which sodium penetrates by facilitated diffusion (Cereijido, Herrera, Flanigan & Curran, 1964; Biber & Curran, 1970; Biber & Sanders, 1973; Erlij & Smith, 1973) and one represented by the basal and lateral membranes which are impermeable to sodium but permeable to potassium (Koefoed-Johnsen & Ussing, 1958; Ussing, 1960; Ussing & Winhager, 1964; Farquhar & Palade, 1965). On these membranes an ATPase controlling $Na^+$ absorption to the internal medium is thought to be localized (Farquhar & Palade, 1965). At which of these levels does harmaline exert its effect? We consider it unlikely that it acts directly on the sodium pump by stimulating an ATPase. Canessa *et al.* (1973) have shown that harmaline (5 mM) inhibits the (Na-K) ATPase activities of various membranes. On the other hand there are some indications that its activating action is due to a reaction at the external barrier of the sodium transport compartment. Thus: (1) sodium transport only increases when harmaline is added to the external face of the preparation, (2) the SCC response to addition of harmaline is extremely rapid, and the reversal after washing is also rapid.

Amiloride reduces sodium transport by blocking the entrance of $Na^+$ into the transport compartment (Biber, 1971). In the present experiments amiloride completely stopped the stimulatory effect of harmaline. This suggests that the additional current recorded in the presence of harmaline passes by the same 'blockable channels' as that present in control conditions.

Ouabain inhibits the $Na^+$ transport by acting mainly on the active pump. When this is totally inhibited by ouabain (SCC = 0) experiments showed that the active transepithelial flux can no longer be stimulated by harmaline. If the SCC is partially reduced by ouabain, harmaline stimulates $Na^+$ net transport in proportion to the SCC. These experiments support the suggestion (Rick, Dörge & Nagel, 1975) that the sodium pump is not the factor limiting transepithelial $Na^+$ transport.

The stimulatory effects on sodium transport of the indole alkaloids used in the above experiments can be compared with the effects of certain imidazolines such as 2 guanidinbenzimidazole (GBI) or phentolamine. Thus, Zeiske & Lindeman (1974) and Garcia-Romeu

**Table 4** Unidirectional sodium fluxes before and after addition of harmaline (5 mM) to the internal solution

| | | | *Control* | *Harmaline (internal) (5 mM)* | *Harmaline (internal) and amiloride (external, 50 μM)* |
|---|---|---|---|---|---|
| A | ($n$ = 13) | $J_{in}$ | 2054 ± 350 | 585 ± 186 | |
| | | SCC | 1946 ± 324 | 256 ± 82 | |
| | | $J_{out}$ | 108 | 329 | |
| | ($n$ = 6) | $J_{in}$ | 2798 ± 534 | 897 ± 257 | 1326 ± 606 |
| | | SCC | 2546 ± 490 | 331 ± 169 | 62 ± 37 |
| | | $J_{out}$ | 252 | 566 | 1264 |
| B | ($n$ = 12) | $J_{in}$ | 1666 ± 214 | 2518 ± 342 | |
| | | SCC | 1556 ± 191 | 228 ± 126 | |
| | | $J_{out}$ | 110 | 2290 | |
| | ($n$ = 7) | $J_{in}$ | 1525 ± 328 | 2487 ± 613 | 2499 ± 670 |
| | | SCC | 1443 ± 295 | 305 ± 190 | 74 ± 75 |
| | | $J_{out}$ | 82 | 2182 | 2425 |

In a third period the fluxes were measured in the presence of harmaline as before but with the addition of amiloride (50 μM) to the external medium. Harmaline period started 90 min after addition of alkaloid. The 25 skins tested fall into 2 groups: (A) harmaline reduces Na influx, (B) does not. All fluxes in nEq $h^{-1}$ $cm^{-2}$. Number of animals in parentheses.

(1974) have shown that GBI in the external medium increases sodium transport by affecting the permeability of the external barrier of the transport compartment. The curves of SCC changes, their times of response and reversibility after GBI treatment are all comparable with those after harmaline treatment.

The *in vivo* crayfish gill reacts differently from the frog skin to harmaline in the external medium. In fact, the $Na^+$ transport of the gill is inhibited by the hallucinogen (Ehrenfeld & Garcia-Romeu, 1975) as it also is by GBI (unpublished results). As the effective dose is low (47% inhibition of influx with 5 μM harmaline and 80% with 0.2 mM) and the effect reversible, it is probable that the inhibitory action of harmaline in the crayfish is a result of interaction with sodium transport sites at the external face and not by way of an ATPase (Ehrenfeld & Garcia-Romeu, 1975). These substances, which possess nitrogen-containing heterocyclic nuclei, may be assumed to interfere with the passage of sodium at the external barrier. Their inhibitory or stimulatory actions are probably related to the nature of the apical membrane and unconnected with any action on a transport ATPase. This interpretation is in agreement with the conclusions of Sepulveda & Robinson (1974).

The addition of harmaline to the internal medium at a low concentration (0.1 mM) has little effect on the SCC. At higher concentrations (5 mM) the SCC falls progressively, and washing only slowly reverses this effect. This SCC diminution was noted by Canessa *et al.* (1973) with harmaline concentrations comparable with those used here (1 to 5 mM). Although under normal conditions the SCC represents the $Na^+$ net flux, under the influence of a drug this need not necessarily be the case. An SCC of zero may result from a variation of either of the unidirectional fluxes. It is essential to know these fluxes in order to interpret the results. Table 4 shows that addition of harmaline to the internal medium gave variable values for influx although all skins were treated in the same way. The results were therefore separated into two groups according to whether harmaline inhibited (Group A) or not (Group B) the sodium influx. In neither group did the epithelium produce a sodium net flux: the effluxes increased and became equal to the influxes and the addition of amiloride (50 μM) did not inhibit the sodium influx. Canessa *et al.* (1973) showed on different preparations that harmaline inhibits (Na-K) ATPases at concentrations higher than 1 mM. The inability of the epithelium to maintain a sodium net flux could be interpreted as being due to an interference of harmaline with a transport ATPase. The persistence of unidirectional influxes after harmaline treatment in one group of skins is not inconsistent with the possibility of the Na pump being inhibited. The increase of the sodium efflux and the presence of an influx insensitive to amiloride suggest that harmaline induces sites for sodium transfer other than those taken by the ion under normal conditions. It is possible that the hallucinogen increases intercellular sodium movements, perhaps by increasing the permeability of the tight junctions.

This work was supported by grants from the Department de Biologie du C.E.A., D.G.R.S.T. (contrat no. 73-7-1520) and C.N.R.S. (Physiologie des membranes, Equipe de recherche associée).

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*(Received May 14, 1976.*
*Revised July 26, 1976)*

*Br. J. Pharmac.* (1977), **59**, 123–128

# PULMONARY METABOLISM OF BRADYKININ ANALOGUES AND THE CONTRIBUTION OF ANGIOTENSIN CONVERTING ENZYME TO BRADYKININ INACTIVATION IN ISOLATED LUNGS

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1 The activity and pulmonary metabolism of two peptides, 7-homo Pro-bradykinin and 8-homo Phe-bradykinin were studied in isolated systems.

2 Both analogues were about 50–70 times less active than bradykinin on the guinea-pig ileum and 70–160 times less active on isolated strips of cat terminal ileum.

3 The action of both analogues on guinea-pig ileum was potentiated (2.5–3.0 fold) by a bradykinin potentiating peptide ($BPP_{9a}$) but less so than the action of bradykinin (4–5 fold).

4 Like bradykinin, the 8-homo Phe analogue was extensively inactivated (>90%) in a single passage through the pulmonary circulation of guinea-pig or rat isolated lungs and this inactivation was prevented by pre-treatment of the lungs with $BPP_{9a}$.

5 The 7-homo Pro analogue was inactivated to a lesser degree in guinea-pig lungs (58%) and in rat lungs (89%) and its inactivation was not affected by $BPP_{9a}$.

6 It is concluded that the 8-homo Phe analogue is a substrate for the dipeptidylcarboxypeptidase (angiotensin I converting enzyme) of lung, whereas the 7-homo Pro analogue is not a substrate.

7 There is about four times as much dipeptidylcarboxypeptidase activity in guinea-pig isolated lungs as there is in rat isolated lungs.

## Introduction

Bradykinin is extensively inactivated during passage through the pulmonary circulation by the action of peptidases. The original findings *in vivo* (Ferreira & Vane, 1967) were extended using isolated lungs of various species. For example, in rat isolated lungs, bradykinin perfused through the pulmonary circulation suffers hydrolysis at several bonds (Figure 1; Ryan, Roblero & Stewart, 1970). Since the hydrolysis of any peptide bond of bradykinin leads to its virtual inactivation (Suzuki, Abiko, Endo, Kameyama, Sasaki & Nabeshima, 1969), any one of the cleavages shown in Figure 1 could represent the action of the fastest acting peptidase and all the other cleavages due to other peptidases would be relatively unimportant in terms of the pulmonary inactivation. One of the pulmonary bradykininases, angiotensin converting enzyme (dipeptidylcarboxypeptidase, E. C. 3. 4. 15. 1) has been well characterized (Soffer, 1976) but its importance relative to the other pulmonary bradykininases has not been assessed. Since two recently synthesized analogues, 7-homo Pro-bradykinin and 8-homo Phe-bradykinin, were said to be resistant to hydrolysis by converting enzyme (Ondetti & Engel, 1975), it was decided to investigate their metabolism relative to that of bradykinin in isolated lungs.

## Methods

Isolated lungs of rats and guinea-pigs of either sex were perfused via the pulmonary artery with oxygenated Krebs solution at 37°C as described earlier (Bakhle, Reynard & Vane, 1969). The composition of the Krebs solution was (mM): $NaHCO_3$ 25, NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2 and glucose 5.6. The lung perfusate was directed over isolated smooth muscle preparations for bioassay and the contractions of the assay tissues recorded via isotonic transducers coupled to a multi-channel Watanabe recorder. The assay tissues were pieces of guinea-pig ileum, and longitudinal strips of cat

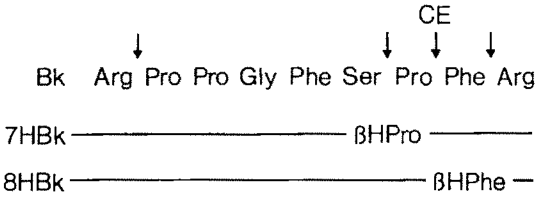

**Figure 1** Structure of bradykinin and the two $\beta$-homo-analogues. The arrows show the points of cleavage in bradykinin perfused through rat isolated lungs (Ryan *et al.*, 1970). CE = angiotensin converting enzyme.

terminal ileum (Alabaster & Bakhle, 1972); they responded to injections of bradykinin of 2–50 ng. The assay tissues were also constantly superfused (0.1 ml/min) with a mixture of antagonists (methysergide, 0.56 µM; mepyramine, 0.35 µM; hyoscine, 0.33 µM; phenoxybenzamine, 0.33 µM; propranolol, 7.5 µM; the final concentrations are given).

The drugs used were: 2-mercaptoethanol (Sigma); methysergide bimaleate (Sandoz); mepyramine hydrochloride (May & Baker); hyoscine hydrobromide (BDH); phenoxybenzamine hydrochloride (Smith, Kline & French); (±)-propranolol hydrochloride (ICI); bradykinin (Sandoz); 7-$\beta$-homo Pro-bradykinin (SQ 22516), 8-$\beta$-homo Phe-bradykinin (SQ 22515) and Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, $BPP_{9a}$ (SQ 20881) (Dr Ondetti, Squibb Institute for Medical Research, Princeton, New Jersey, U.S.A.).

## Results

### *Activity of bradykinin analogues on isolated tissues*

The first experiments were designed to assess the potency of the analogues relative to the parent peptide on the two isolated smooth muscle preparations that were to be used later to study the pulmonary metabolism of the peptides. 7-Homo Pro-bradykinin, 8-homo Phe-bradykinin, and bradykinin were assayed on the same preparations, guinea-pig ileum and cat terminal ileum (Figure 2). The potency of the analogues was expressed as bradykinin equivalents (Table 1). On the cat terminal ileum the $\beta$-homo analogues had different potencies whereas on the guinea-pig ileum they were effectively equipotent.

The responses of the guinea-pig ileum to bradykinin are potentiated by the nonapeptide, $BPP_{9a}$ (Ferreira, Greene, Alabaster, Bakhle & Vane, 1970). The effects of a constant infusion of $BPP_{9a}$ on the responses of the guinea-pig ileum to bradykinin and the two analogues are shown in Table 2. The $\beta$-homo analogues were potentiated to a lesser degree than bradykinin. The responses of the cat terminal ileum to the $\beta$-homo analogues, like those to bradykinin (Alabaster & Bakhle, 1972), were not potentiated by $BPP_{9a}$.

### *Inactivation in isolated lungs*

Like bradykinin, both $\beta$-homo analogues were inactivated on a single passage through guinea-pig and

**Table 1** Agonist potency on isolated smooth muscles of 7-homo Pro- and 8-homo Phe-bradykinin relative to bradykinin

| | | *Bradykinin equivalent (ng) on* | |
|---|---|---|---|
| *Analogue* | *Dose (µg)* | *guinea-pig ileum* | *cat terminal ileum* |
| | | *(mean ± s.e.)* | |
| 7-homo Pro | 0.5 | 7.7 ± 0.8<br>(10)† | 6.5 ± 0.5<br>(10) |
| | 1.0 | 19.7 ± 1.8<br>(10) | 13.7 ± 1.1<br>(14) |
| | 2.0 | 33.3 ± 2.4<br>(8) | 29.0 ± 1.3<br>(9) |
| 8-homo Phe | 0.5 | 8.0 ± 0.9<br>(12) | — |
| | 1.0 | 14.1 ± 0.9<br>(10) | 6.1 ± 0.3<br>(9) |
| | 2.0 | 25.3 ± 3.8<br>(10) | 11.1 ± 0.8<br>(15) |
| | 3.0 | — | 19.6 ± 2.1<br>(10) |

† Numbers in parentheses refer to number of experiments.

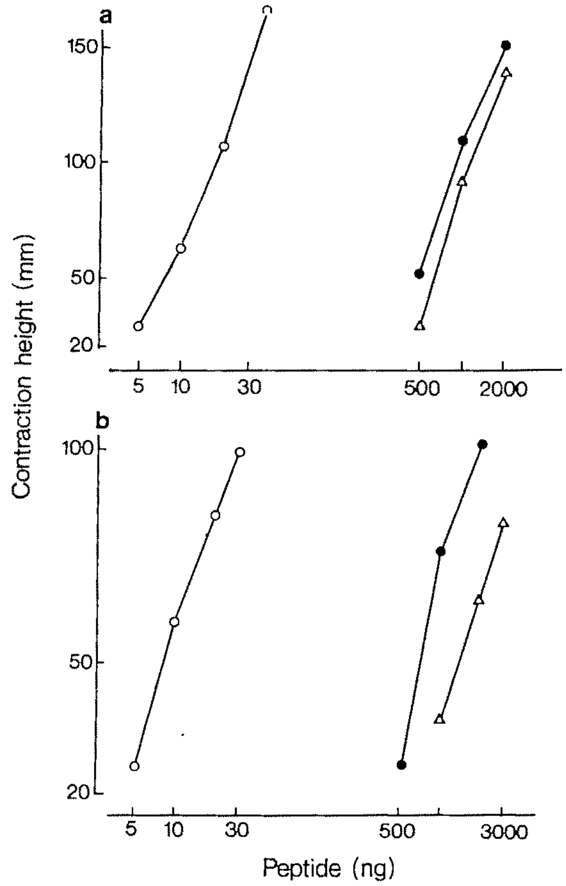

**Figure 2** Dose-response relationships for bradykinin (○), 7-homo Pro-bradykinin (●) and 8-homo Phe-bradykinin (△) on (a) the guinea-pig isolated ileum and (b) cat terminal ileum. The tissues were superfused with Krebs solution (8 ml/min) and the peptides administered by injection (0.1–0.2 ml) into the superfusing fluid.

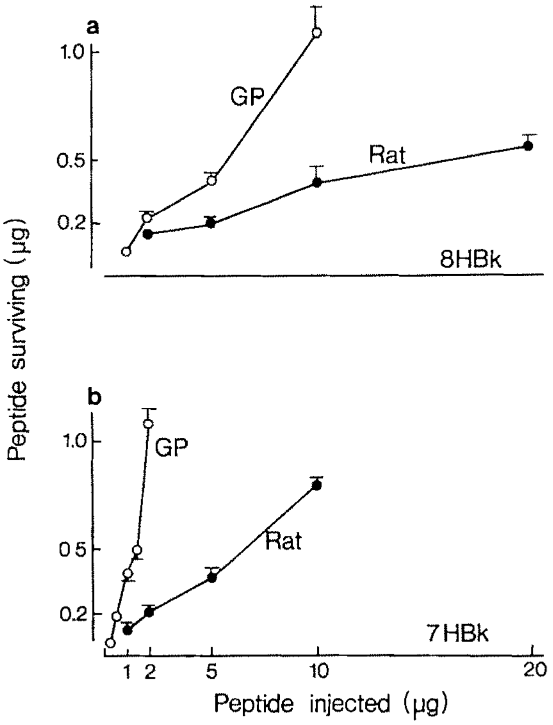

**Figure 3** Survival of (a) 8-homo Phe-bradykinin (8HBk) and (b) 7-homo Pro-bradykinin (7HBk) after a single passage through the pulmonary circulation of isolated lungs of guinea-pig and rat. The amount of peptide surviving a single passage through the pulmonary circulation was measured by bioassay and each point represents the mean of at least four assays. Vertical lines show s.e. means.

rat isolated lungs, though there were important quantitative differences. The relationship between the amount of β-homo analogue injected into the pulmonary artery and the amount of activity surviving passage through the pulmonary circulation of guinea-pig and rat lungs is shown in Figure 3. In both species, 7-homo Pro-bradykinin showed a higher survival than 8-homo Phe-bradykinin. Furthermore, although the 8-homo Phe analogue, like bradykinin itself, was over 90% inactivated in both guinea-pig and rat lungs, over 40% of the 7-homo Pro analogue survived in guinea-pig lungs, more than 4 times its survival in rat lungs.

The bradykinin potentiating peptide, $BPP_{9a}$, is also a specific inhibitor of the dipeptidylcarboxypeptidase that hydrolyses angiotensin I and bradykinin. Figure 4 shows that in guinea-pig lung, an infusion of $BPP_{9a}$ (100 ng/ml) increased survival of bradykinin and 8-homo Phe-bradykinin five-fold ($P < 0.05$), whereas the already high survival of 7-homo Pro-bradykinin was not affected. At the higher concentration of $BPP_{9a}$ (500 ng/ml) there was a small further increase in survival of bradykinin and its 8-homo Phe analogue and a non-significant change in the survival of 7-homo Pro-bradykinin. In rat lung, survival of all three peptides is much lower than in guinea-pig lung but $BPP_{9a}$ again increased survival of bradykinin and 8-homo Phe-bradykinin ($P < 0.05$), whereas survival of the 7-homo Pro analogue was not significantly changed even at the higher concentration of $BPP_{9a}$ (500 ng/ml).

## Discussion

The change in the molecule brought about by the substitution of a β-homo-amino acid for the normal α-amino acid is comparatively small, extending the peptide backbone by one carbon atom in the chain of eight peptide links, but it reduces the potency of both analogues as agonists on intestinal smooth muscle. The analogues were probably acting via bradykinin receptors since the dose-response relationships were roughly parallel to those for bradykinin and the potentiating peptide $BPP_{9a}$ also potentiated the analogues. The potentiation of bradykinin by the peptides from *Bothrops jararaca*, of which $BPP_{9a}$ is one, was originally attributed to inhibition of bradykinin degradation by tissues (Ferreira, 1965), but subsequently a direct interaction of the potentiator with the receptor has been postulated (Camargo & Ferreira, 1971). If this is so, then the changes in the receptor brought about by $BPP_{9a}$ are not as effective for the analogues as they are for bradykinin. Furthermore, although the analogues are equally active on the guinea-pig ileum, the 8-homo Phe analogue was more potentiated by $BPP_{9a}$ than the 7-homo Pro analogue.

The analogues were both designed to be resistant to the major bradykinin hydrolysing enzyme in lung, the dipeptidylcarboxypeptidase or angiotensin converting enzyme. This enzyme (see Figure 1) is known to cleave bradykinin initially at the $Pro^7$-$Phe^8$ bond (Dorer, Kahn, Lentz, Levine & Skeggs, 1974) and therefore substitutions at $Pro^7$ or $Phe^8$ were expected to interfere with the action of the enzyme. Furthermore, $BPP_{9a}$ which protects bradykinin from inactivation by dipeptidylcarboxypeptidase should have no effect on the inactivation, if any, of the analogues. Even under conditions of maximal inhibition of converting enzyme, some inactivation of the analogues would be expected, as the other bradykinin hydrolysing peptidases (see Figure 1) would not be inhibited.

The expected resistance to dipeptidylcarboxypeptidase was found only with 7-homo Pro-bradykinin. This analogue was more resistant than bradykinin to inactivation in the isolated lung and the inactivation that did occur was unaffected by concentrations of $BPP_{9a}$ that gave near maximal protection to bradykinin. 8-Homo Phe-bradykinin was, like bradykinin, rapidly inactivated by the lung and protected by $BPP_{9a}$. These results lead to the conclusion that 7-homo Pro-bradykinin was not a substrate or at best a very poor substrate, for converting enzyme, whereas 8-homo Phe-bradykinin was a substrate.

These conclusions are at variance with those of Ondetti & Engel (1975). They found that a purified converting enzyme preparation from rabbit lung liberated the C-terminal dipeptide Phe-Arg from bradykinin, but not from the two analogues and concluded that neither analogue was a substrate for converting enzyme. However, the results of their experiments *in vivo* were not compatible with this conclusion, as 8-homo Phe-bradykinin was a less potent vasodepressor than bradykinin and its effects, like those of bradykinin, were enhanced by a previous

**Table 2** Potentiation by $BPP_{9a}$ of the contractor effects of bradykinin and two analogues on guinea-pig isolated ileum

| | *Dose of agonist peptide* | *Dose equivalent after treatment*† | *Potentiation (fold)* |
|---|---|---|---|
| Bradykinin | 1 | 5 (2) | 5.0 |
| (ng) | 2 | 8.1 ± 1.2 (4) | 4.0 |
| | 5 | 23.6 ± 3.0 (8) | 4.7 |
| | 10 | 40.2 ± 2.7 (8) | 4.0 |
| 7-homo Pro | 0.2 | 0.5 ± 0.1 (5) | 2.5 |
| (μg) | 0.5 | 1.1 ± 0.1 (8) | 2.3 |
| | 1.0 | 2.4 ± 0.1 (8) | 2.4 |
| | 2.0 | 4.7 ± 0.2 (8) | 2.4 |
| 8-homo Phe | 0.2 | 0.6 ± 0.1 (7) | 3.1 |
| (μg) | 0.5 | 1.6 ± 0.1 (8) | 3.1 |
| | 1.0 | 2.8 ± 0.2 (4) | 2.8 |

The $BPP_{9a}$ in a final concentration of 100 ng/ml was superfused continuously over the isolated tissues for at least 15 min before, and during the administration of the agonist peptides.

†Dose equivalent is the amount of agonist that would be required to give an equivalent contraction on untreated tissue; thus 1 ng of bradykinin causes a contraction of treated ileum equivalent to that given by 5 ng of bradykinin on the ileum before treatment (mean ± s.e. mean).

Number of experiments is given in parentheses.

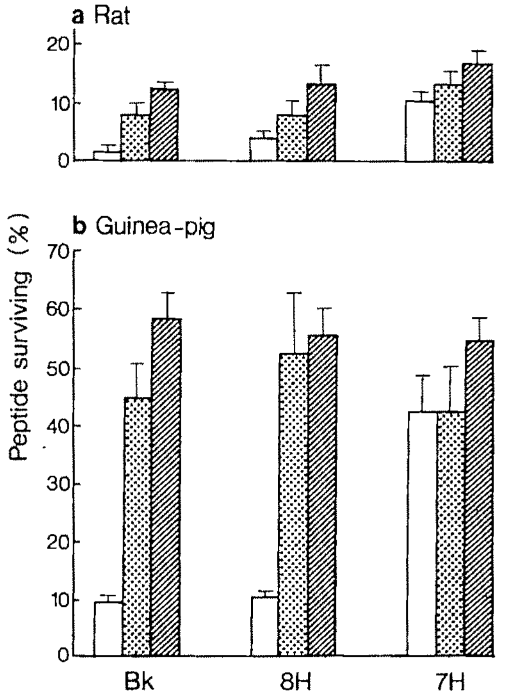

Figure 4 Effect of $BPP_{9a}$ 100 ng/ml (dotted columns) and 500 ng/ml (hatched columns) on survival of bradykinin (Bk), 7-homo Pro-bradykinin (7H) and 8-homo Phe-bradykinin (8H) in (a) rat and (b) guinea-pig isolated lungs. The height of the columns represents the mean (±s.e. mean) survival in 6 or more assays, expressed as a percentage of the amount injected into the pulmonary circulation. The open columns represent survival under control conditions. $BPP_{9a}$ increased survival of bradykinin and the 8-homo analogue ($P<0.05$) but not that of the 7-homo Pro-bradykinin ($P>0.1$).

injection of $BPP_{9a}$. 7-Homo Pro-bradykinin, however, was 30 times as potent as the 8-homo Phe analogue and its effects were not enhanced by $BPP_{9a}$.

These differences between the properties of the analogues *in vivo* agree well with the findings that 8-homo Phe-bradykinin, but not 7-homo Pro-bradykinin, was a substrate for converting enzyme. My findings also suggest that, *in vivo*, relatively more 7-homo Pro-bradykinin survives the pulmonary, blood and peripheral converting enzymes and is recirculated. This would explain the longer hypotension (2.1 min) produced by 7-homo Pro-bradykinin, compared with bradykinin or 8-homo Phe-bradykinin (0.5–0.4 min) (Ondetti & Engel, 1975).

The present study also provides information on the level of angiotensin converting enzyme activity in isolated lungs. The inactivation of 7-homo Pro-bradykinin can be taken as an indication of the proportion of converting enzyme relative to the other bradykinin hydrolysing peptidases; thus in guinea-pig lung about half, and in rat, nearly all of the total bradykinin hydrolysing activity was due to peptidases other than converting enzyme. These results also suggest that these other peptidases inactivate bradykinin and the two analogues at very similar rates, possibly because these enzymes are acting at bonds not involving the $Pro^7$ or $Phe^8$ residues (see Figure 1). This indirect estimate of a low level of converting enzyme activity in rat isolated lungs agrees with the direct estimation of the conversion of angiotensin I to angiotensin II in isolated lungs (Bakhle, Reynard & Vane, 1969) and with results obtained *in vivo* (Kreye & Gross, 1971) suggesting that in the rat, conversion of angiotensin I in the lung is less than in the peripheral circulation. This large difference (four-fold) in converting enzyme activity in the lungs of two common laboratory animals means that results obtained in one species cannot be compared directly with those obtained in the other species.

I thank Christine Pepper for excellent technical assistance and the M.R.C. for financial support. I am grateful to Sandoz for the gift of bradykinin and to Dr Ondetti, Squibb Institute for Medical Research, Princeton, New Jersey, U.S.A. for the bradykinin analogues, 7-β-homo Pro-bradykinin and 8-β-homo Phe-bradykinin and for $BPP_{9a}$.

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*(Received June 2, 1976.*
*Revised July 24, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 129–133

# A COMPARISON OF THE EFFECTS OF SODIUM THIOCYANATE AND DANTROLENE SODIUM ON A MAMMALIAN ISOLATED SKELETAL MUSCLE

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**1** A combination of electrical and pharmacological stimulation has been used to compare the effects of sodium thiocyanate and dantrolene sodium on the excitation-contraction coupling (ECC) mechanism of the mouse soleus muscle.

**2** Thiocyanate prolonged the 'active state' after electrical stimulation and increased the response to 8 mM caffeine and 80 mM KCl. Dantrolene had an opposite effect to thiocyanate on all the indices studied.

**3** It is concluded that the mechanism of action of dantrolene is by inhibition of the release of the calcium ions involved in the ECC mechanism, probably at the level of the transverse tubules.

## Introduction

Dantrolene sodium is a recently discovered skeletal muscle relaxant (Snyder, 1967). It has already been found useful in the treatment of skeletal muscle spasticity (Dykes, 1975), and is thought to have a unique mechanism of action by interfering with the excitation-contraction coupling (ECC) mechanism.

There is considerable interest in elucidating in detail its exact mode of action. Firstly, it would seem to have a precise action on a single step of the ECC process, probably by interfering with the calcium release mechanism which couples the sarcolemmal action potential to the contractile process (Ellis & Carpenter, 1972; Hainaut & Desmedt, 1974; Putney & Bianchi, 1974). It therefore provides a potentially useful tool for studying this mechanism, about which at present very little is known. Secondly, it has been found to be successful in the treatment of the syndrome of malignant hyperpyrexia occurring under general anaesthesia (Harrison, 1975). This syndrome has been shown to be caused by an abnormal sensitivity of skeletal muscle to various drugs, but particularly general anaesthetic agents (Moulds & Denborough, 1974). Exposure of isolated malignant hyperpyrexia muscle from either susceptible humans (Moulds & Denborough, 1974) or pigs (Anderson & Jones, 1976) to halothane produces an abnormal contracture, probably by an excessive release of the calcium ions involved in the ECC mechanism and dantrolene has been shown to prevent this abnormal contracture (Anderson & Jones, 1976). Therefore a more detailed knowledge of its mechanisms of action would also almost certainly help to elucidate the underlying defect in muscle predisposing to malignant hyperpyrexia.

Many agents are known to influence the ECC mechanism in skeletal muscle, but probably most is known about the twitch potentiating effect of the lyotropic anions (Sandow, 1965). Like dantrolene, they probably also act on a single step of the ECC process and prolong the 'active state' during which calcium ions are released. Therefore, in order to learn more about the mechanism of action of dantrolene, a detailed comparison has been made between its effects on mammalian isolated skeletal muscle and those of the most potent of the lyotropic anions, the thiocyanate ion.

## Methods

The soleus muscles from young adult Balb/c mice of either sex were used. Each tendon was tied with black linen thread and attached to two small rings and the muscle suspended vertically in a 20 ml tissue bath. It was maintained at 37°C by a Churchill pump and the bathing fluid had the following composition (mM): $Na^+$ 137.4, $K^+$ 5.4, $Ca^{2+}$ 2.5, $Mg^{2+}$ 1.2, $Cl^-$ 131.6, $SO_4^{2-}$ 1.2, $H_2PO_4^-$ 1.2, $HCO_3^-$ 15.0 and glucose 11.5. $O_2$ containing 5% $CO_2$ was bubbled through the solution which then had a pH between 7.2 and 7.4.

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Isometric tension was measured with a Statham force transducer and recorded on a Devices high speed recorder. The resting tension was adjusted to give the maximum twitch and was usually 2–3 grams. Curare, 25 µg/ml, was sometimes added to the bath solution, but it had no effect on the responses being tested so was usually omitted. Electrical stimulation was performed through two thin platinum foil electrodes placed in contact with each end of the muscle preparation, using a Grass S44 stimulator set at supramaximal voltage (usually 30 V) and with pulses of 0.5 ms duration.

Dantrolene sodium was suspended as a slurry in 0.1 N NaOH in a concentration of 1 mg/ml and the appropriate amount added to the bath solution to give a final concentration of 3 µg/ml (approx. 10.5 µmol/litre). Caffeine (Sigma) was dissolved in the bath solution (not containing calcium) to make a concentration of 100 mM and the appropriate volume added to the bath to give a final concentration of 8 mM. Potassium chloride (KCl) was made up as a stock solution of 2 M and the appropriate volume added to the bath to give a final concentration of 80 mM.

## Results

In order to study the ECC mechanism as closely as possible, a combination of electrical and pharmacological tests was used. The following measurements were therefore made on normal solei, and on solei exposed for at least 10 min to either dantrolene 3 µg/ml or NaCNS 20 mM: twitch height, tetanus height, the half-life of the exponential phase of the relaxation from a tetanus, the frequencies required to produce a tetanus, the height and time to peak of the contracture produced by 80 mM KCl, and the height of the contracture produced after 10 min by 8 mM caffeine.

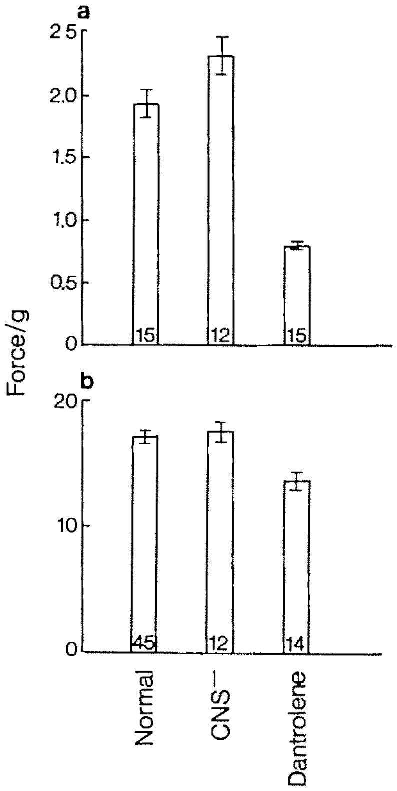

**Figure 1** The effect of dantrolene and thiocyanate (CNS⁻) on (a) the twitch and (b) maximum tetanus of the mouse isolated soleus muscle. The numbers of solei tested in each case are shown at the base of the column, and error bars are s.e. mean.

**Table 1** Twitch height, maximum tetanus height and relaxation half-life in normal solei, solei exposed to NaCNS (20 mM) and solei exposed to dantrolene sodium (3 µg/ml)

| | *Normal solei* | *Solei in NaCNS* | *Solei in dantrolene* |
|---|---|---|---|
| Twitch height (g) | 1.93±0.11<br>(15) | 2.32±0.15<br>$P<0.05$<br>(12) | 0.80±0.03<br>$P<0.001$<br>(15) |
| Tetanus height (g) | 16.11±0.44<br>(45) | 16.49±0.79<br>NS<br>(12) | 13.61±0.71<br>$P<0.005$<br>(14) |
| Tetanus relaxation $T\frac{1}{2}$ (ms) | 16.72±0.44<br>(45) | 16.56±0.73<br>NS<br>(14) | 16.66±0.75<br>NS<br>(12) |

Results are mean±s.e. in each case. Number of solei tested in each case are given in parentheses.
*P* values are result of statistical analysis compared with normal solei (unpaired Student's *t* test) in each case.

### (a) *Electrical stimulation*

A comparison between the effects of dantrolene and NaCNS on the height of the twitch and a tetanus is given in Table 1 and shown diagrammatically in Figure 1. It can be seen that, as has been previously reported for amphibian muscle (Ellis & Carpenter, 1972), in this mammalian muscle preparation dantrolene had a greater inhibitory effect on the twitch than on the tetanus, and also thiocyanate enhanced the twitch whilst having no effect on the tetanus. Table 1 also shows that despite their marked and opposite effects on the twitch height, neither of these agents had a significant effect on the time course of the relaxation from a tetanus.

It was found that when the force developed (as a percentage of the maximum) by the muscle was plotted against the logarithm of the frequency of electrical stimulation, then a sigmoid curve typical of a pharmacological dose-response curve was obtained. As shown in Figure 2, this curve was shifted markedly to the right (i.e. much higher frequencies of stimulation were required for tetanic tension to develop) by dantrolene, and was shifted to the left (i.e. tetanic tension developed at lower frequencies) by NaCNS.

### (b) *Pharmacological stimulation*

The addition of either KCl (80 mM) or caffeine (8 mM) to the bath solution produced in normal solei typical contractures, illustrations of which are shown in Figure 3. The comparison between the effects of dantrolene and of NaCNS on these contractures is given in Table 2, and typical examples are illustrated in Figure 3. It can be seen that dantrolene inhibited both the contracture produced by KCl (80 mM) and that produced by caffeine (8 mM), while conversely NaCNS potentiated both of the contractures.

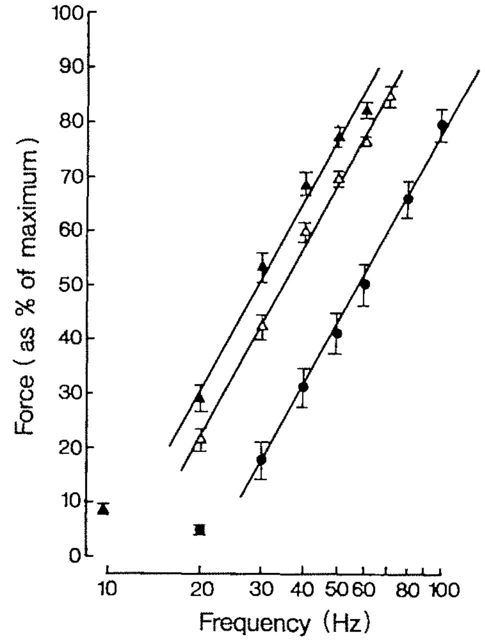

**Figure 2** Plots of the force developed by the muscle (as a percentage of the maximum) against the logarithm of the frequency of electrical stimulation. Each point is the mean of measurements made on twelve normal solei (△), twelve solei in NaCNS 20 mM (▲) and five solei in dantrolene sodium 3 µg/ml (●). Vertical lines show s.e. mean.

## Discussion

The results described in this paper show that dantrolene has an effect on a mammalian isolated muscle preparation similar to that which it has been

**Table 2** Contractures produced by KCl (80 mM) or caffeine (8 mM) in normal solei, solei exposed to NaCNS (20 mM), and solei exposed to dantrolene sodium (3 µg/ml)

| | | *Normal solei* | *Solei in NaCNS* | *Solei in dantrolene* |
|---|---|---|---|---|
| KCl 80 mM | Height of contracture (g) | $3.75 \pm 0.13$<br>(10) | $9.21 \pm 0.34$<br>$P < 0.001$<br>(5) | $2.00 \pm 0.24$<br>$P < 0.001$<br>(4) |
| | Time to peak of contracture (s) | $24.6 \pm 2.5$<br>(10) | $67.0 \pm 4.26$<br>$P < 0.001$<br>(4) | $42.0 \pm 2.55$<br>$P < 0.001$<br>(5) |
| Caffeine 8 mM | Height of contracture (g) | $2.08 \pm 0.45$<br>(10) | $3.40 \pm 0.59$<br>$P < 0.01$<br>(5) | $0.81 \pm 0.13$<br>$P < 0.05$<br>(5) |

Results are mean ± s.e. mean in each case. Number of solei tested in each case are given in parentheses. $P$ values are result of statistical analysis compared with normal solei (unpaired Student's $t$ test) in each case.

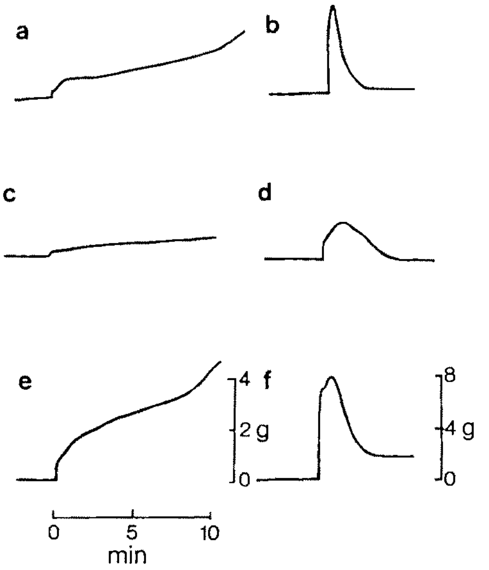

**Figure 3** Examples of contractures obtained by the addition of caffeine 8 mM (left hand column) or KCl 80 mM (right hand column) to normal solei (a, b), solei in dantrolene sodium 3 µg/ml (c, d) and solei in NaCNS 20 mM (e, f). The horizontal time scale for all contractures is as shown for (e), but note that the vertical force scale (in g) is as shown for (e) in all the contractures except (f), where the scale is halved.

reported to exert on frog isolated muscle – i.e. it causes a marked reduction in twitch height but a much smaller reduction in the height of a tetanus. More importantly however, dantrolene has been shown to have directly opposite effects from another agent which influences the ECC mechanism, the thiocyanate ion.

The lyotropic anions are generally considered to have a specific action on the ECC mechanism by prolonging the 'active state' produced by the sarcolemmal action potential during which calcium ions are released into the myoplasm. This action is almost certainly exerted superficially on the sarcolemma, and it has been suggested that it is mediated by a change in the electrical field surrounding the T-tubules by virtue of the greater adsorption of the lyotropic anions to the sarcolemmal membrane (Bianchi, 1968; Chapman, 1969).

The effects of the thiocyanate ion described in this paper are compatible with this mechanism, and they will be considered in turn.

An agent which increased the amount of calcium released by each action potential would be expected to enhance a single twitch, but not a maximal tetanus, the height of which is probably determined by the limits of the contractile elements themselves rather than the availability of calcium ions. The relaxation from a tetanus is also probably determined either by the rate of re-uptake of the activating calcium ions, or more likely by the actual rate of disassociation of the actomyosin crossbridges. Whichever of these mechanisms is responsible, neither would be expected to be influenced by an agent which merely increased the amount of calcium released by a single action potential.

The frequency of stimulation required to produce a tetanus is more complex, potentially being a function of at least three and probably more different processes, these being the amount of calcium released by the repetitive stimuli, the rate of re-uptake of that calcium by the sarcoplasmic reticulum, and the rate of disassociation of the actomyosin crossbridges. However, if an agent increased the amount of calcium released per impulse without affecting any of the other processes, then the muscle would develop tension at a lower frequency of stimulation and that is what was found. Furthermore, the 'frequency-response' curve in the presence of thiocyanate was parallel to the normal curve also suggesting that the presence of the thiocyanate caused a constant amount of extra calcium to be released by each impulse.

The mechanism of the contracture produced by 80 mM KCl is by an artificial and persistent depolarization of the sarcolemmal membrane, thus simulating the transient depolarization produced by an action potential. Any agent which changed the electrical properties of the membrane such that more calcium ions were released by depolarization would therefore also be expected to cause potentiation of the contracture produced by 80 mM KCl.

However, the contracture produced by caffeine (8 mM), is more complex than that produced by KCl (80 mM). It is independent of the membrane polarity and has been shown to be due in part to a release of the calcium ions involved in ECC coupling and in part to an inhibition of their re-uptake by the sarcoplasmic reticulum (Weber & Herz, 1968). The major source of the calcium ions causing a caffeine contracture is generally considered to be from the sarcoplasmic reticulum and not the sarcolemma. But the lyotropic anions have also been found to enhance the caffeine contracture in frog muscle (Foulks, Perry & Sanders, 1971), and the enhancement of the caffeine contracture produced by thiocyanate in this study was relatively modest. It may therefore well have been due to an enhancement of the sarcolemmal contribution to the calcium release.

The above discussion shows that the effects of sodium thiocyanate are compatible with an enhancement of the release of calcium ions early in the ECC process, and probably at the transverse tubular level. A similar argument suggests that the effects of dantrolene, being the opposite of those of thiocyanate, are compatible with an inhibition of the release of the calcium ions involved in the ECC process. Some direct evidence to support this conclusion is provided by the work of Putney & Bianchi (1974) and Hainaut & Desmedt (1974) who found that dantrolene had no effect on the resting influx of Ca into frog muscle, but decreased the influx associated with a twitch.

It is therefore concluded that in a mammalian muscle dantrolene acts on the ECC process by reducing the amount of calcium released by the sarcolemmal action potential. Moreover the inhibition probably occurs early in the coupling process, most probably at the site of the transverse tubules.

The author is an overseas fellow of the Australian NH and MRC and is grateful to Dr D.A. Jones, Dr R.H.T. Edwards and Professor C.T. Dollery for advice and encouragement, and to Dr A.W. Castellion of the Norwich Pharmacal Company for the supply of dantrolene.

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*(Received June 2, 1976.*
*Revised July 26, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 135–140

# DIFFERENTIATION OF METABOLIC ADRENOCEPTORS

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1 Cardiovascular and metabolic responses to intravenous infusion of isoprenaline were measured in fasted, anaesthetized cats.

2 Isoprenaline (0.2 μg $kg^{-1}$ $min^{-1}$ for 15 min) decreased diastolic blood pressure and increased heart rate, blood glucose, blood lactate and plasma free fatty acids.

3 Oxprenolol (0.5 mg/kg) antagonized all cardiovascular and metabolic effects of isoprenaline non-selectively.

4 Para-oxprenolol (0.25 mg/kg) and practolol (4 mg/kg) antagonized the effects of isoprenaline on heart rate and free fatty acids selectively.

5 H 35/25 ((1-(4-methylphenyl)-2-isopropyl aminopropanol) hydrochloride, 3 mg/kg) antagonized the effects of isoprenaline on blood pressure, glucose and lactate selectively.

6 It is concluded that metabolic adrenoceptors are differentiated into subtypes similar to those mediating cardiostimulation and vasodilatation.

## Introduction

It has been proposed that $\beta$-adrenoceptors mediating pharmacological responses to sympathomimetic agonists are differentiated into two subtypes designated '$\beta_1$' and '$\beta_2$' (Lands, Arnold, McAuliff, Luduena & Brown, 1967). This classification is based on the relatively selective activity of certain agonists in producing these responses. Thus, adrenoceptors on which noradrenaline has strong activity, such as those mediating cardiostimulation, have been assigned to the $\beta_1$ subtype. Conversely, adrenoceptors on which noradrenaline has weak activity, including those mediating vasodilatation and bronchodilatation, have been assigned to the $\beta_2$ subtype. For each response, the activity of noradrenaline has been assessed in comparison with that of other agonists, primarily adrenaline and isoprenaline, which appear to have strong activity on both the $\beta_1$ and $\beta_2$ subtypes of adrenoceptor.

The selective agonist activity of noradrenaline has also been used to classify $\beta$-adrenoceptors mediating metabolic responses. Thus, due to the strong activity of noradrenaline on lipolytic adrenoceptors mediating release of free fatty acids (FFA), these adrenoceptors have been assigned to the $\beta_1$ subtype (Lands *et al.*, 1967). Conversely, liver and muscle glycogenolytic adrenoceptors mediating release of glucose and lactate, respectively, have been assigned to the $\beta_2$ category due to the relatively weak activity of noradrenaline in producing these responses (Arnold, McAuliff, O'Connor & Brown, 1968).

Metabolic responses to adrenaline and isoprenaline have also been compared with those produced by selective non-catecholamine agonists. Tazolol, or ITP, selectively produces cardiostimulation (Strosberg & Roszkowski, 1972); this agonist increases plasma FFA but does not increase glucose or lactate (Lockwood & Lum, 1974). Tazolol is therefore a presumably $\beta_1$ selective agonist which supports the Lands' classification of $\beta$-adrenoceptors. However, the actions of presumably $\beta_2$ selective agonists are less consistent with this classification. Thus, salbutamol selectively produces bronchodilatation (Cullum, Farmer, Jack & Levy, 1969) yet this agonist is more active in producing increases in plasma FFA and less active in producing increases in blood lactate (Kelly & Shanks, 1975) than would be expected in a selective $\beta_2$ agonist.

In the present study, an attempt has been made to provide evidence regarding differentiation of metabolic adrenoceptors by the use of $\beta$-adrenoceptor antagonists which are presumed to be non-selective or selective for either cardiac or vascular $\beta$-adrenoceptors. Cardiovascular and metabolic responses to intravenous infusion of isoprenaline have been measured in control animals. These responses have been compared to those observed in animals pretreated with practolol, an antagonist which selectively blocks isoprenaline-induced cardiostimulation (Dunlop & Shanks, 1968) and with H 35/25, an antagonist which selectively blocks isoprenaline-induced vasodilatation (Levy & Wilkenfeld, 1969). Responses to isoprenaline have also been examined in animals pretreated with oxprenolol or para-oxprenolol, antagonists which have been reported to have dissimilar selectivity for cardiovascular $\beta$-adrenoceptors (Vaughan Williams, Bagwell & Singh, 1973).

## Methods

Fasted adult cats (2.0–4.1 kg) of either sex were anaesthetized with intravenous pentobarbitone sodium 30 mg/kg. Supplemental intravenous doses of pentobarbitone were administered as needed to maintain anaesthesia. Both vagosympathetic nerve trunks were ligated and severed in the neck. Systolic and diastolic blood pressures (BP) were monitored via a Statham PT 06 transducer from a catheter inserted into a carotid artery. Heart rate was determined by counting the number of systolic peaks on the blood pressure recording. Recordings were made on a Hewlett Packard Model 7700 polygraph. A femoral artery and vein were cannulated to obtain arterial blood samples and to administer drugs, respectively. The trachea was cannulated and animals were allowed to respire spontaneously. Following completion of surgical procedures, heparin sodium (250 units/kg) was administered intravenously to prevent clotting of blood samples.

Arterial blood glucose levels were determined by the *o*-toluidine method of Dubowski (1962). Arterial blood lactate levels were determined by the lactate dehydrogenase method of Marbach & Weil (1967). Arterial plasma FFA were measured by the copper complexation method of Duncombe (1964).

The following drugs were used: (±)-isoprenaline hydrochloride (Winthrop Laboratories, Inc.), oxprenolol hydrochloride (Ciba-Geigy, Ltd.), para-oxprenolol hydrochloride (Ciba-Geigy, Ltd.), practolol (Imperial Chemical Industries, Ltd.) and H 35/25 (1-(4-methylphenyl)-2-isopropyl aminopropanol) hydrochloride, Axel Kistner AB). The dosages of isoprenaline and practolol are in terms of the bases; the dosages of oxprenolol, para-oxprenolol, H 35/25 are in terms of the salts. Isoprenaline was administered by continuous intravenous infusion; infusions were performed with a Harvard Model 975 infusion pump calibrated to deliver a volume of 0.5 ml of isoprenaline solution in 0.9% w/v NaCl solution (saline) per minute. Oxprenolol, para-oxprenolol, practolol and H 35/25 were administered over a 5 min period which was completed 15 min before beginning infusion of isoprenaline. Arterial blood samples of 2.5 ml each were removed for analysis immediately before administration of isoprenaline and $\beta$-adrenoceptor antagonists and at 5, 10 and 15 min after beginning isoprenaline infusion.

Statistical analyses were performed using Student's *t* test for group comparison or the *t* test for paired data as appropriate. A *P* value of less than 0.05 was considered to be significant.

## Results

### *Cardiovascular responses to isoprenaline*

Heart rate and diastolic BP were recorded just before (time 0) and at 5, 10 and 15 min after beginning isoprenaline infusion. Isoprenaline significantly increased heart rate and decreased diastolic blood pressure; these effects persisted throughout the period of infusion but were maximal at 5 min after beginning isoprenaline administration (Table 1).

### *Metabolic responses to isoprenaline*

Blood glucose, blood lactate and plasma FFA levels were determined in samples taken just before (time 0) and at 5, 10 and 15 min after beginning isoprenaline infusion. Isoprenaline significantly increased glucose, lactate and FFA levels; these effects were progressive throughout the period of infusion and therefore were maximal at 15 min after beginning isoprenaline administration (Table 1).

### *Cardiovascular and metabolic responses to $\beta$-adrenoceptor antagonists*

Cardiovascular and metabolic parameters were measured just before and at 15 min after administration of oxprenolol, para-oxprenolol, practolol or H 35/25. The doses of antagonists used were those found to produce significant antagonism of at least one of the cardiovascular responses to isoprenaline infusion. Oxprenolol, para-oxprenolol and practolol produced small but significant increases in blood glucose (Table 2). As shown in Table 2, para-oxprenolol was the only antagonist which produced significant changes in diastolic blood pressure, blood lactate and plasma FFA while oxprenolol was the only antagonist which produced a significant change in heart rate.

### *Effect of antagonists on cardiovascular responses to isoprenaline*

Heart rate and diastolic BP were recorded at time 0, which was 15 min after administration of antagonists and just before beginning isoprenaline, and at 5, 10 and 15 min after beginning isoprenaline infusion. Maximal changes in heart rate and diastolic BP produced by isoprenaline in animals pretreated with antagonists were compared with maximal changes produced in non-pretreated controls (Table 3). Oxprenolol non-selectively antagonized both the increase in heart rate and the decrease in blood pressure produced by isoprenaline in controls. In contrast to this, para-oxprenolol and practolol selectively antagonized the isoprenaline-induced increase in heart rate while H 35/25 selectively

**Table 1** Cardiovascular and metabolic responses to isoprenaline (0.2 μg kg$^{-1}$ min$^{-1}$)

| *Time* | *Heart rate (beats/min)* | *Diastolic BP (mmHg)* | *Blood glucose (mg %)* | *Blood lactate (mg %)* | *Plasma FFA (mEq/l)* |
|---|---|---|---|---|---|
| 0 | 112 ± 10 | 88 ± 8 | 85 ± 5 | 18.6 ± 1.3 | 0.44 ± 0.03 |
| 5 | 162 ± 9* | 51 ± 7* | 126 ± 7* | 23.8 ± 0.9* | 0.57 ± 0.05* |
| 10 | 161 ± 9* | 62 ± 10* | 156 ± 7* | 31.1 ± 0.7* | 0.71 ± 0.10* |
| 15 | 159 ± 8* | 66 ± 9* | 167 ± 3* | 33.1 ± 0.6* | 0.77 ± 0.09* |

Number of animals = 7. Time represents time in minutes after beginning isoprenaline infusion.
* Significantly different from initial (time 0) values ($P < 0.05$).

**Table 2** Cardiovascular and metabolic responses to β-adrenoceptor antagonists

| *Antagonist* | n | Δ *Heart rate (beats/min)* | Δ *Diastolic BP (mmHg)* | Δ *Blood glucose (mg%)* | Δ *Blood lactate (mg%)* | Δ *Plasma FFA (mEq/l)* |
|---|---|---|---|---|---|---|
| Oxprenolol (0.5 mg/kg) | 3 | −28 ± 10* | −5 ± 3 | 6 ± 1* | 1.6 ± 0.9 | −0.01 ± 0.01 |
| Para-oxprenolol (0.25 mg/kg) | 5 | 3 ± 5 | −8 ± 3* | 28 ± 3* | 1.5 ± 0.5* | 0.03 ± 0.01* |
| Practolol (4 mg/kg) | 6 | −13 ± 9 | −8 ± 4 | 15 ± 4* | 1.0 ± 0.78 | 0.003 ± 0.008 |
| H 35/25 (3 mg/kg) | 6 | −5 ± 3 | −3 ± 3 | 1 ± 1 | 0.1 ± 0.1 | 0.01 ± 0.01 |

*n* = number of animals. Δ represents change from initial values.
* Significant change from initial values ($P < 0.05$).

**Table 3** Effect of antagonist pretreatment on cardiovascular and metabolic responses to isoprenaline (0.2 μg kg$^{-1}$ min$^{-1}$)

| *Pretreatment* | n | Δ *Heart rate (beats/min)* | Δ *Diastolic BP (mmHg)* | Δ *Blood glucose (mg%)* | Δ *Blood lactate (mg%)* | Δ *Plasma FFA (mEq/l)* |
|---|---|---|---|---|---|---|
| None (controls) | 7 | 50 ± 6 | −37 ± 4 | 82 ± 5 | 14.5 ± 4.0 | 0.33 ± 0.08 |
| Oxprenolol (0.5 mg/kg) | 3 | 11 ± 1* | −3 ± 2* | 28 ± 5* | 2.7 ± 1.4* | 0.01 ± 0.01* |
| Para-oxprenolol (0.25 mg/kg) | 5 | 14 ± 2* | −42 ± 8 | 77 ± 5 | 13.2 ± 0.6 | 0.04 ± 0.02* |
| Practolol (4 mg/kg) | 6 | 20 ± 1* | −40 ± 4 | 79 ± 5 | 11.9 ± 1.9 | 0.02 ± 0.01* |
| H 35/25 (3 mg/kg) | 6 | 43 ± 3 | −2 ± 4* | 16 ± 6* | 0.9 ± 0.8* | 0.28 ± 0.07 |

*n* = number of animals. Δ represents maximal change from initial values.
* Significantly different from response to isoprenaline in Controls ($P < 0.05$).

antagonized the isoprenaline-induced decrease in diastolic blood pressure.

### *Effect of antagonists on metabolic responses to isoprenaline*

Blood glucose, blood lactate and plasma FFA levels were determined in samples taken at time 0, which was 15 min after administration of antagonists and just before beginning isoprenaline infusion, and 5, 10 and 15 min after beginning isoprenaline infusion. Maximal changes in blood glucose, blood lactate and plasma FFA produced by isoprenaline in animals pretreated with antagonists were compared with maximal changes produced in non-pretreated controls (Table 3). Oxprenolol non-selectively antagonized all metabolic responses to isoprenaline. In contrast to this, para-oxprenolol and practolol selectively antagonized the isoprenaline-induced increase in plasma FFA while H 35/25 selectively antagonized the isoprenaline-induced increases in blood glucose and blood lactate.

## Discussion

The metabolic adrenoceptors mediating isoprenaline-induced increases in blood glucose, blood lactate and plasma FFA appear to consist exclusively of $\beta$-adrenoceptors. Accordingly, it has been established that these metabolic responses to isoprenaline can be completely blocked by the $\beta$-adrenoceptor antagonist, propranolol (Barrett & Cullum, 1968). Unlike isoprenaline, other sympathomimetic agonists such as adrenaline are associated with an $\alpha$-adrenoceptor-mediated inhibition of pancreatic insulin release which presumably contributes to the increase in blood glucose produced by these agonists (Altszuler, Gottlieb, Steel & Bjerknes, 1974). Although the increase in blood glucose produced by isoprenaline appears to be due primarily to stimulation of liver glycogenolysis, it is possible that this response is enhanced by simultaneous stimulation of muscle glycogenolysis. This is consistent with the observation that elevation of blood lactate (by i.v. infusion of sodium lactate) significantly increases blood glucose (Miller, Issekutz, Paul & Rodahl, 1964). This is probably due to the increased availability of lactate for liver gluconeogenesis. On the other hand, elevation of blood lactate or glucose may diminish increases in plasma FFA due to stimulation of lipolytic adrenoceptors by facilitating re-esterification to triglycerides (Miller *et al.*, 1964). This interaction may partly account for the relatively large increases in plasma FFA characteristic of noradrenaline, an agonist which produces relatively small increases in blood lactate or glucose.

Previous attempts to differentiate metabolic adrenoceptors have been concerned primarily with differences between activities of noradrenaline and other sympathomimetic agonists in producing responses attributable to glycogenolysis or lipolysis. For example, noradrenaline has been demonstrated to have very weak activity compared to isoprenaline in producing stimulation of muscle glycogenolysis (Fleming & Kenny, 1964). Since many responses to noradrenaline can be potentiated by pretreatment of animals with neuronal uptake blockers such as cocaine, it may be argued that weak activity of noradrenaline relative to other catecholamines is due to rapid inactivation of noradrenaline by neuronal uptake. However, except for increases in blood glucose, metabolic responses to noradrenaline are only slightly potentiated by cocaine pretreatment and such potentiation requires doses of cocaine greater than those necessary to potentiate other noradrenaline responses such as vasoconstriction (Hardman & Mayer, 1965). Furthermore, neuronal uptake inactivation does not explain the relatively strong activity of noradrenaline compared to other catecholamines in producing increases in plasma FFA (Barrett, 1965). In general, studies with noradrenaline and other selective agonists appear to support the concept that metabolic adrenoceptors are heterogeneous and are differentiated into subtypes.

Relatively few studies have used selective antagonism of metabolic responses to sympathomimetic agonists to differentiate metabolic adrenoceptors. Practolol, a presumably $\beta_1$ selective antagonist, selectively inhibited adrenaline-induced increases in plasma FFA in one study but the dose of practolol which was used did not alter adrenaline-induced increases in heart rate (Cash, Woodfield & Allan, 1970) and therefore blockade of the 'cardiac' $\beta$-adrenoceptor subtype is problematical. Butoxamine, an antagonist which selectively antagonizes isoprenaline-induced vasodilatation (Parratt & Wadsworth, 1970), also antagonizes isoprenaline-induced increases in glucose and lactate (Salvadore, April & Lemberger, 1966). However, butoxamine has also been reported to block increases in plasma FFA produced by sympathomimetic agonists (Colville, Lindsay & Burns, 1965) and this action would not be expected from a presumably $\beta_2$ selective antagonist. Blockade of metabolic responses to sympathomimetic agonists by other selective $\beta$-antagonists such as para-oxprenolol or H 35/25 does not appear to have been investigated.

In the present investigation, non-selective antagonism of cardiostimulant and vasodilator responses to isoprenaline by oxprenolol was accompanied by antagonism of all metabolic responses measured. In contrast to this, selective antagonism of the cardiostimulant responses to isoprenaline by para-oxprenolol or practolol was accompanied by selective antagonism of the effect of isoprenaline on plasma FFA. The apparent failure of practolol pretreatment to antagonize glycogenolytic adrenoceptors is consistent with the results of Pogatsa, Kaldor & Vizi (1970) who found that practolol, unlike the non-selective $\beta$-antagonist propranolol, did not inhibit stimulation of glycogenolysis or glucose release by isoprenaline in perfused rat liver. The selective antagonism of the vasodilator response to isoprenaline by H 35/25 was accompanied by selective antagonism of the effects of isoprenaline on blood glucose and lactate. The apparent failure of H 35/25 to antagonize lipolytic adrenoceptors is in contrast to the blockade of FFA release reported for butoxamine and may reflect a greater degree of selectivity of H 35/25 for $\beta_2$-adrenoceptors compared to butoxamine.

The results of the present investigation support the concept that metabolic adrenoceptors generally classified as '$\beta$' are heterogeneous and can be further differentiated into subtypes comparable to those proposed by Lands *et al.* (1967). Thus, lipolytic adrenoceptors appear to resemble adrenoceptors mediating cardiostimulant responses to sympathomimetic agonists whereas glycogenolytic adrenoceptors appear to resemble adrenoceptors mediating vasodilator responses. The results of this study do not necessarily confirm the $\beta_1$, $\beta_2$, classification of Lands *et al.* since other tissues may contain $\beta$-adrenoceptors which are not equivalent to those mediating either cardiostimulation or vasodilatation. However, the ability of selective $\beta$-adrenoceptor antagonism to dissociate lipolysis from other adrenergic responses has potential clinical value in view of evidence that increases in FFA may facilitate production of cardiac arrhythmias by adrenoceptor agonists (Kurien, Yates & Oliver, 1971). Due to the apparent differentiation of adrenoceptors, it is possible that selective lipolytic blockade can be achieved without interfering with desired responses (such as bronchodilatation) to therapeutic doses of sympathomimetic drugs.

The authors would like to express their appreciation to Prof. H. Brunner and Dr K. Scheibli of Ciba-Geigy, Ltd. (Basle) for the generous gift of oxprenolol and paraoxprenolol and to M. Wharmby of ICI Ltd. for the generous gift of practolol.

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*Br. J. Pharmac.* (1977), **59**, 141–145

# INTERACTION OF COCAINE WITH CHLORDIAZEPOXIDE ASSESSED BY MOTOR ACTIVITY IN MICE

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1 Effects of a range of doses of cocaine and chlordiazepoxide given separately and as mixtures were determined on the spontaneous locomotor activity of mice.

2 Cocaine increased locomotor activity (walking) during 3 or 5 min trials in a dose-related manner.

3 Chlordiazepoxide had little effect on the total amount of locomotor activity except for depression at very high doses. A lower dose of chlordiazepoxide increased activity at the beginning of the trials only.

4 Mixtures containing certain doses of cocaine and chlordiazepoxide increased locomotor activity to a much greater extent than cocaine alone. This high level of activity was manifested throughout 5 min trials.

5 This action of cocaine is similar to that of amphetamine.

## Introduction

The behavioural effects of cocaine have been studied less intensively than those of the synthetic central nervous system stimulants such as amphetamine. In common with amphetamines, cocaine can facilitate locomotor activity and induce stereotyped behaviours, can either increase or decrease rates of operant responding, and can serve as a powerful positive reinforcer (Woods & Downs, 1973). There is also evidence that cocaine can substitute for amphetamine as a discriminative stimulus in rats trained to respond differentially according to their drug state (Huang & Ho, 1974). However, cocaine has often been found to be less potent and to have a shorter duration of action than amphetamine, and it may also differ in its interactions with iproniazid, α-methyltyrosine and haloperidol (Smith, 1965; Simon, Sultan, Chermat & Boissier, 1972). Amphetamine can induce rotational behaviour in rats with unilateral lesions of the nigrostriatal dopamine pathway, but cocaine does so only after the administration of a monoamine oxidase inhibitor (Christie & Crow, 1973).

Experiments have been carried out to examine further the behavioural profile of action of cocaine by testing its interaction with chlordiazepoxide. When rodents are placed in an unfamiliar environment, mixtures of amphetamine with chlordiazepoxide or barbiturates can induce much more locomotor activity (walking) than the constituent drugs given separately (Rushton & Steinberg, 1963, 1966; Rushton, Steinberg & Tomkiewicz, 1973). A failure to find hyperactivity produced by mixtures of cocaine and chlordiazepoxide would add support to the view that there are significant differences between the actions of cocaine and amphetamine.

## Methods

### *Animals*

Female albino mice weighing 16–25 g were used throughout. They were housed in colony cages containing 8–10 mice and had unlimited access to food and water.

### *Locomotor activity*

A mouse was placed in a rectangular chamber (26 x 18 x 10 cm) constructed of black Perspex, with a clear Perspex lid. Test chambers with dimensions of this order can elicit an activity level sufficiently low for further facilitation by drugs to be clearly demonstrable (Kršiak & Janků, 1971). The chamber was illuminated by a 60 W lamp at a height of 1 metre. The amount of time spent walking was recorded cumulatively on an electronic timer, directly controlled by an observer. Rearing onto the hind feet was not included. The duration of the trial was either 5 min (experiment 1) or 3 min (experiments 2 and 3) and each mouse was used once only. In experiment 1 only, the activity scores were also printed automatically every minute. Inter-observer correlations were determined on several occasions and were considered to indicate a satisfactory degree of reproducibility (e.g. $r=0.94$, d.f. 14, $P<0.001$).

### *Experiment 1*

Thirty mice were allocated to 4 treatment groups by a randomization procedure ($n = 7$–$8$). The groups were injected with either cocaine (20 mg/kg), chlordiazepoxide (15 mg/kg), a mixture of both drugs, or isotonic saline. These doses were selected on the basis of previous work with the drugs given separately (e.g. Rushton *et al.*, 1973; Christie & Crow, 1973).

### *Experiment 2*

In order to test a range of doses of cocaine and chlordiazepoxide, 80 mice were allocated to 10 groups by a randomization procedure ($n = 8$). Three groups received cocaine (7.5, 15.0 or 30.0 mg/kg), 3 groups received chlordiazepoxide (10.0, 20.0 or 40.0 mg/kg), and 3 groups received mixtures of both drugs such that the ratio of the doses (cocaine:chlordiazepoxide) was held constant at 0.75:1. The tenth group received isotonic saline.

### *Experiment 3*

In order to test a wider range of doses and dose-ratios, 250 mice were allocated to 25 groups by a randomization procedure ($n = 10$). A range of doses of cocaine (4.0, 8.0, 16.0 or 32.0 mg/kg) and chlordiazepoxide (4.0, 8.0, 16.0 or 32.0 mg/kg) were studied when administered separately and in all combinations. The ratio of cocaine:chlordiazepoxide in the mixtures studied varied therefore from 0.125:1 to 8.0:1. One group of mice received isotonic saline.

### *Drugs*

Cocaine hydrochloride (B.P.) or chlordiazepoxide hydrochloride (Librium, Roche) were dissolved in saline and injected intraperitoneally in a volume of 0.2 ml per mouse 20 min before locomotor activity was assessed. Mixtures of cocaine and chlordiazepoxide were given in a single injection and all doses were calculated as salts.

### *Statistical analysis*

The results were analysed by single factor and two factor analyses of variance, and by Dunnett's $t$ test for multiple comparisons with a control group (Winer, 1971). Locomotor activity scores were subjected to square root transformation to stabilize variances (cf. Kršiak, Steinberg & Stolerman, 1970).

## **Results**

### *Experiment 1*

The mean motor activity scores for control mice receiving saline declined slightly but not significantly during the course of the 5 min trial. Cocaine (20 mg/kg) slightly increased the mean activity scores ($F = 5.41$, d.f. 1,104, $P < 0.05$) and from Figure 1 it can be seen that this effect was essentially constant throughout the trial. Chlordiazepoxide (15 mg/kg) increased activity significantly during the first minute only ($t = 2.21$, d.f. 13, $P < 0.05$); this was followed by a decline in activity at a rate which was significantly greater than that for saline controls ($t = 3.91$, d.f. 13, $P < 0.01$). Figure 1 also shows that the mean activity scores for mice receiving the mixture of cocaine and chlordiazepoxide were consistently higher than those for the mice receiving cocaine alone ($F = 25.0$, d.f. 1,104, $P < 0.001$). This high level of activity was maintained throughout the trial, but since the effect of the mixture was evident within the first 3 min, trials of this length were used subsequently.

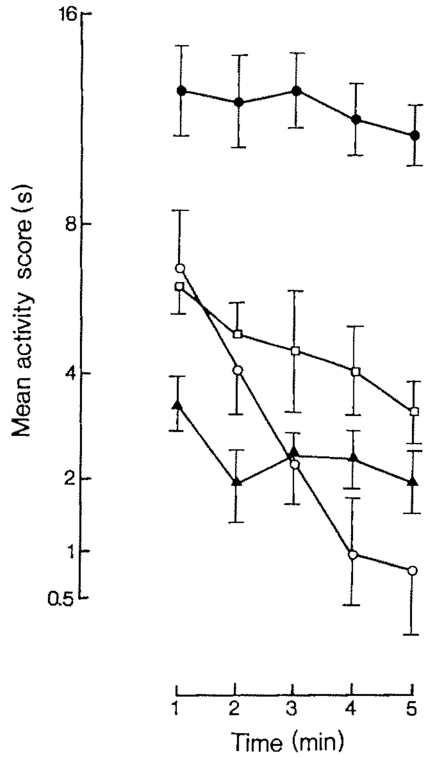

**Figure 1** Activity of mice influenced by cocaine (20 mg/kg, □) and chlordiazepoxide (15 mg/kg, ○) given separately and as a mixture (●). Each activity score is the mean for 7–8 mice and the vertical bars indicate one standard error on each side of the mean (square root scale). Some bars have been omitted for clarity. Controls received saline (▲).

### *Experiment 2*

Administration of cocaine alone increased the mean activity scores ($F = 3.29$, d.f. 3.28, $P < 0.05$), although

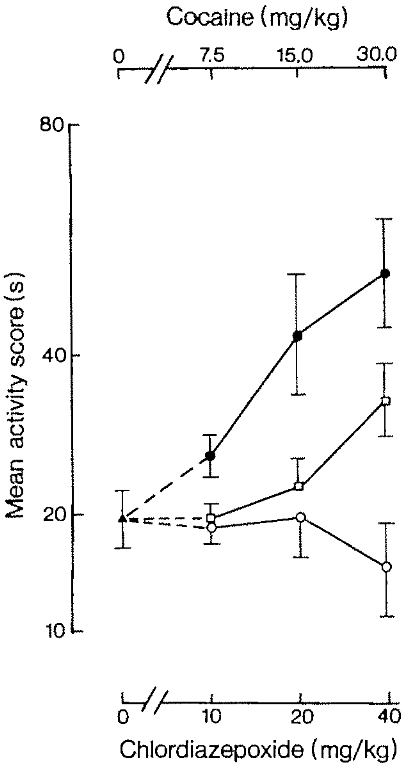

**Figure 2** Activity of mice influenced by cocaine (□) and chlordiazepoxide (○) given separately at 3 doses of each, and in mixtures (●) of the same 3 doses in a constant ratio of 0.75:1 ($n=8$). (▲) Control mice.

this effect was not marked except at the highest dose used (30 mg/kg). Chlordiazepoxide alone had no significant effects on the mean activity scores ($F<1$, d.f. 3,28); a depressant tendency at the highest dose was not statistically significant but it was noted that some mice showed characteristic periods of total immobility. Figure 2 shows that mixtures of cocaine and chlordiazepoxide significantly increased activity scores as compared with saline ($F=5.27$, d.f. 3,28, $P<0.01$). A two-factor (3 x 3) analysis of variance on the 9 groups of mice receiving drug treatments confirmed an overall difference between drugs ($F=24.6$, d.f. 2,63, $P<0.001$) and it was further shown that mixtures of cocaine and chlordiazepoxide yielded significantly greater mean activity than cocaine alone ($F=10.7$, d.f. 1,63, $P<0.01$), which in its turn yielded greater activity than chlordiazepoxide alone ($F=4.09$, d.f. 1,63, $P<0.05$). The overall differences between dose levels and the drug x doses interaction were not significant.

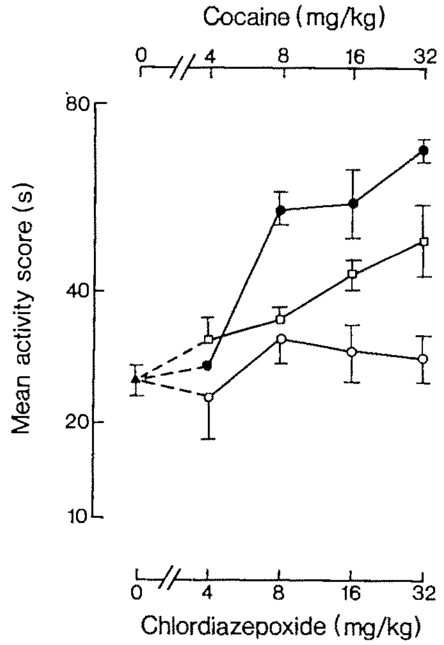

**Figure 3** Activity of mice influenced by cocaine (□) and chlordiazepoxide (○) given separately at 4 doses of each, and in mixtures (●) of the same doses in a constant ratio of 1:1 ($n=10$). (▲) Control mice.

### *Experiment 3*

The results for cocaine and chlordiazepoxide given separately and in mixtures at a dose ratio of 1:1 are shown in Figure 3 in a format which facilitates comparison with experiment 2 (Figure 2). Cocaine alone increased the mean activity scores in a dose-related manner ($F=5.65$, d.f. 4,45, $P<0.01$). Chlordiazepoxide alone had no significant effect ($F<1$, d.f. 4,45) nor was there a noteworthy trend across doses with this drug. Figure 3 also shows that in adequate doses, mixtures of the two drugs increased activity significantly above that after the administration of cocaine alone ($F=9.68$, d.f. 1,83, $P<0.01$). Cocaine yielded activity greater than that after chlordiazepoxide alone ($F=10.6$, d.f. 1,83, $P<0.01$). It may be noted that this pattern of results is essentially similar to that for experiment 2.

The complete results of experiment 3 are represented in Figure 4 as a response surface, and the following statistical comparisons refer to differences between mixtures and the constituent doses of cocaine given alone. Chlordiazepoxide administered in mixtures with the lowest dose of cocaine (4 mg/kg) had no significant effect. However, in mixtures with cocaine (8 mg/kg), chlordiazepoxide significantly

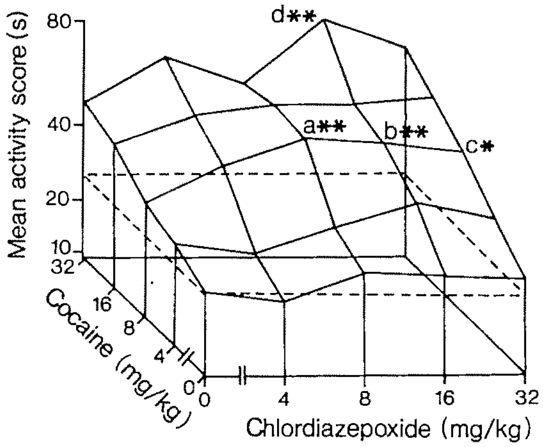

**Figure 4** Response surface for doses of cocaine and chlordiazepoxide, showing the mean activity scores for groups of 10 mice given the drugs separately or in mixtures. Points are indicated where mice receiving mixtures were significantly more active than mice receiving the constituent dose of cocaine only (*$P<0.05$; **$P<0.01$). The dashed line represents the activity scores for mice receiving saline only.

increased the mean activity scores when given in doses of 8 mg/kg ($P<0.01$), 16 mg/kg ($P<0.01$) and 32 mg/kg ($P<0.05$). These effects can be seen as a peak approximately in the centre of the response surface and are marked as (a), (b) and (c) in Figure 4. Chlordiazepoxide did not increase activity significantly in mixtures with cocaine (16 mg/kg). The effects of chlordiazepoxide mixed with the highest dose (32 mg/kg) of cocaine did not show a good dose-response relationship, but chlordiazepoxide (16 mg/kg) yielded higher activity than cocaine alone ($P<0.01$). This can be seen as a second peak (d) in the response surface. The variance of the activity scores was greatest with the highest dose of cocaine, contributing to the lack of a good dose-response relationship.

## Discussion

Mixtures of cocaine and chlordiazepoxide can greatly increase the coordinated locomotor activity of mice in an unfamiliar environment. Cocaine given alone also increases activity, but to a lesser extent. Earlier work has shown that mixing amphetamine with chlordiazepoxide can also produce very high activity in both rats (Rushton & Steinberg, 1966) and mice (Rushton *et al.*, 1973) and therefore, in this respect the behavioural effects of cocaine and amphetamine appear to be similar.

Increased activity scores due to cocaine (without chlordiazepoxide) are consistent with previous work (Rossum, 1964; Smith, 1965). Amphetamine does not usually produce very marked increases in walking when this component of activity is distinguished from stereotyped responses such as stepping on the spot and head-shaking (Rushton & Steinberg, 1963; Kršiak *et al.*, 1970). Typically, high doses of amphetamine tend to suppress walking whereas in the present experiments, even large amounts of cocaine increased walking, but directly comparable data for amphetamine would be required to differentiate the two drugs on this basis. It is known that both cocaine and amphetamine can suppress operant behaviour when administered in sufficiently high doses (Smith, 1964). The effect of chlordiazepoxide (without cocaine) on mice in the present experiments was strikingly similar to that reported previously in rats, in the sense that an initially increased level of activity was followed by a sharp decline accompanied by periods of total immobility (Rushton & Steinberg, 1966).

Mixtures of cocaine with chlordiazepoxide yielded higher levels of activity than those produced by either drug given separately and this effect was seen in three separate experiments. Sansone (1975) has found that cocaine (1.0–10.0 mg/kg) in mixtures with chlordiazepoxide (10.0 mg/kg) can facilitate avoidance responding of mice in shuttle-boxes. However, avoidance responding was increased by chlordiazepoxide given separately as well as in mixtures, whereas inter-trial responses were not increased by chlordiazepoxide alone. In detailed studies of amphetamine-barbiturate interactions, it has been reported that apparent potentiation or antagonism may occur depending on which aspect of the performance of a complex task is used to assess the effects of the drugs (Rutledge & Kelleher, 1965; Branch, 1974). How these different interactions may be mediated in the central nervous system remains unclear, especially since little information is available about ways in which the mixtures of drugs influence their distribution and metabolism. Nevertheless, testing for interactions of the type shown can provide an empirical method for characterizing the profile of action of central nervous system stimulants.

The authors wish to thank Professor P.B. Bradley for his encouragement of this work. Chlordiazepoxide was generously supplied by Roche Products Ltd.

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*(Received June 10, 1976.*
*Revised July 6, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 147–156

# THE EFFECTS OF GLUCAGON, SECRETIN, PANCREOZYMIN AND PENTAGASTRIN ON THE HEPATIC ARTERIAL VASCULAR BED OF THE DOG

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**1** The sympathetically-innervated arterial vascular bed of the dog's liver was perfused from a femoral artery. Arterial blood flow and perfusion pressure were monitored continuously and the hepatic arterial vascular resistance (HAVR) calculated from these measurements.

**2** Commercial preparations of secretin, pancreozymin, glucagon and pentagastrin were administered by intra-arterial (i.a.) injection and infusion.

**3** Secretin and pancreozymin by injection caused dose-dependent hepatic arterial vasodilatation, and on a molar basis were both more potent than glucagon or pentagastrin.

**4** Intra-arterial infusions of secretin and pancreozymin caused hepatic arterial vasodilatation at calculated blood concentrations close to those measured under physiological conditions by other investigators. The vasodilatation was of the same duration as that of the hormone infusions.

**5** Pentagastrin by i.a. injection caused dose-dependent hepatic arterial vasodilatation; by i.a. infusion, vasodilatation occurred but there was marked 'escape' from the effects during the continued infusion.

**6** As reported previously, glucagon by injection caused dose-dependent hepatic arterial vasodilatation of long duration; by infusion, glucagon caused vasodilatation that persisted after the cessation of the infusion.

**7** Glucagon infused i.a., inhibited the vasoconstrictor effects of i.a. noradrenaline, over the same range of infusions that caused hepatic arterial vasodilatation.

**8** Secretin or pancreozymin did not antagonize the effects of noradrenaline on the hepatic arterial vascular bed at any doses used.

**9** Pentagastrin did not antagonize the vasoconstrictor effect of noradrenaline whether hepatic arterial vasodilatation resulted from the pentagastrin infusion, or not.

**10** These results are discussed with respect to the possible control of the hepatic arterial vascular bed by gastrointestinal hormones.

## Introduction

The four gastrointestinal hormones pancreozymin, gastrin, secretin and glucagon are secreted from their sources into the portal vein to enter the liver. After passage through the liver and cardiopulmonary circuit they enter the systemic arterial system where, by distribution to the stomach and pancreas, they exert their established actions as secretagogues. Once again they enter the liver in the arterial supply.

Glucagon and secretin both evoke dose-dependent hepatic arterial vasodilatation (Richardson & Withrington, 1976b), and, in addition, glucagon inhibits the hepatic arterial vasoconstrictor actions of noradrenaline, angiotensin and vasopressin (Richardson & Withrington, 1976a), and of adrenaline and 5-hydroxytryptamine (Richardson & Withrington, unpublished).

The present experiments were carried out with three aims: first, to establish the effects of pancreozymin on the hepatic arterial vascular bed and its molar potency in relation to glucagon and secretin; second, to discover whether the hepatic arterial vasoconstrictor response to noradrenaline is affected by secretin and pancreozymin, as in the case of glucagon; and third, to investigate, in addition to the three naturally-occurring hormones, the vascular actions of a synthetic analogue of gastrin, pentagastrin.

The physiological implications of our findings will be discussed, particularly in relation to the reported blood levels of these hormones.

## Methods

Experiments were performed on nine dogs weighing between 10.3 and 16.0 kg (12.3 ± 1.9 kg; mean ± s.d.)

which had not been fed for 24 h prior to the experiments, but had been allowed access to water *ad libitum* throughout this time. Anaesthesia was induced by sodium methohexitone (7.5–10 mg/kg i.v.; Brietal, Lilly) and maintained with $\alpha$-chloralose (50 mg/kg i.v.; Kuhlmann, Paris) and urethane (500 mg/kg i.v., BDH), supplementary doses of chloralose and urethane, in the same proportions, being given as necessary to maintain a constant level of anaesthesia.

The preparations were essentially as described previously (Richardson & Withrington, 1976b, c). Following a midline laparotomy, the hepatic artery was dissected free from its periarterial sympathetic nerves, which were carefully preserved intact. The animals were heparinized (Weddel Pharmaceuticals; 250 iu/kg i.v., followed by 100 iu/kg hourly) and the common hepatic artery cannulated and perfused with blood from the cannulated left femoral artery. The blood flow in this cannula (hepatic arterial blood flow; HABF) was measured with a cannulated flowprobe and electromagnetic flowmeter (Cardiovascular Instruments) and the hepatic arterial perfusion pressure (PP) was measured from a 'T'-piece in the cannula, close to the point of cannulation of the hepatic artery, with a Consolidated Electrodynamics L221 strain gauge transducer. Additional 'T' pieces in this cannula system were used for the intra-arterial (i.a.) administration of vasoactive agents.

When the surgery was complete, the laparotomy incision was closed, and a thermometer inserted into the abdominal cavity; intra-abdominal temperature was maintained at 37–38°C with table heaters and heating lamps. Stable control variables were recorded for at least 20 min prior to the administration of vasoactive agents.

To monitor possible systemic effects of the vasoactive agents which were administered i.a. to the liver, systemic arterial blood pressure was measured from the cannulated right femoral artery with a Statham P23Gb strain gauge transducer, and the heart rate derived electronically with a Devices 4521 ratemeter.

### *Recording of variables*

*Hepatic arterial mean perfusion pressure (PP)* was derived electronically with a Devices 3502 averaging circuit with time constants 0.5, 1.0 or 2.0 s selected appropriately. Both mean and phasic perfusion pressures were recorded continuously.

*Hepatic arterial mean blood flow (HABF)* was derived electronically by passing the phasic waveform through an averaging circuit with a time constant of 0.6 second. Both mean and phasic flow records were displayed continuously.

After appropriate amplification, all recordings were displayed continuously on a Devices M 19 rectilinear recorder.

### *Calculation of results*

*Liver weight* was obtained immediately after each experiment. Values expressed per 100 g refer to this terminal weight of liver.

*Hepatic arterial vascular resistance (HAVR)* was calculated as hepatic arterial mean perfusion pressure (mmHg) divided by hepatic arterial mean blood flow (ml/min, or ml $\text{min}^{-1}$ 100 $\text{g}^{-1}$), and expressed as mmHg $\text{ml}^{-1}$ min, or mmHg $\text{ml}^{-1}$ min 100 g.

*Changes in vascular resistance* were calculated as percentage changes from the control values immediately prior to any procedure, i.e., change in vascular resistance × 100/control vascular resistance.

When the hormones were injected i.a. to the liver, there were large increases in hepatic arterial blood flow, which were accompanied by small reductions in perfusion pressure. The myogenic and hydrostatic changes in hepatic arterial vascular resistance which occur as a result of the reductions in perfusion pressure (Bayliss, 1902; Folkow, 1964) are insignificant compared with the direct effects of the vasoactive substances in these preparations (Richardson & Withrington, 1976a, 1976b).

### *Expression of results*

Except where indicated to the contrary, results are expressed as means ± s.e. means.

Where the term $\text{ED}_{50}$ is used, it indicates the dose of any substance which produced 50% of the maximum effect that could be obtained by progressively increasing i.a. injections of that substance.

The time courses of the responses to i.a. injections of the substances are expressed as the time taken for 50% recovery from the peak effects of selected doses of each substance ($T_{\frac{1}{2}}$). The doses selected for this analysis were just submaximal doses which produced about the same reduction in HAVR for each substance (Richardson & Withrington, 1976b).

The direct effects of i.a. infusions of the agents are expressed as the difference in HAVR during the infusions compared with the mean control vascular resistance before and after each infusion. The control vascular resistances after the infusions did not differ systematically from the control vascular resistances before the infusions, the mean post-infusion vascular resistance being 4.1 ± 2.2% lower than the pre-infusion value.

The influence of i.a. infusions on the responses of the hepatic arterial vasculature to i.a. injections of noradrenaline was also assessed. A test dose of noradrenaline (10.0 µg) which was selected on the basis of previous experiments (Richardson & Withrington, 1976a), produced a repeatable and pronounced increase in HAVR. At least 3 injections of noradrenaline were made before, during and after the infusions of glucagon, secretin, pancreozymin and pentagastrin, and the mean increases in calculated HAVR due to noradrenaline were calculated for each period. The mean increase in HAVR due to the noradrenaline injections during the infusion was then compared to the mean effect of noradrenaline both before and after infusion, and the percentage difference calculated. A positive sign to this percentage difference indicates that the increase in HAVR due to the i.a. injection of noradrenaline during an infusion was greater than the increase in HAVR due to noradrenaline under control conditions, and *vice versa.*

### *Vasoactive substances*

Since pure forms of the hormones are not available in adequate quantities for this type of investigation, commercially-available preparations have been used throughout. The substances used were: glucagon hydrochloride (Lilly), secretin (natural secretin, Boots), pancreozymin (natural pancreozymin, Boots) and the synthetic gastrin pentapeptide analogue, pentagastrin (Peptavlon, ICI). Doses of glucagon and pentagastrin are expressed in μg or ng, and doses of secretin and pancreozymin in Crick-Harper-Raper Units and milliunits (u, mu) where for secretin 1 unit is equivalent to 62.5 ng and for pancreozymin 1 unit is equivalent to 333 ng (manufacturer's data). Molar concentrations were calculated on the basis of molecular weights supplied by the manufacturers: glucagon, 3485; secretin, 3056; pancreozymin, 3883; pentagastrin 767.9. Test doses of (−)-noradrenaline (10.0 μg base; Levophed, Winthrop), were also used.

All vasoactive substances were dissolved in, or diluted from ampoules with 0.9% w/v NaCl solution (saline). Intra-arterial injections were made at a point between the flowprobe and the hepatic arterial cannula, resulting in an injection artifact which was clearly separable from the subsequent response of the preparation (Richardson & Withrington, 1976a, b). Intra-arterial infusions were from a Watson-Marlow MHRE200 pump precalibrated to deliver 1.0 ml/min against pressures in excess of the hepatic arterial perfusion pressure.

The volume of the external circuit was compensated for by an i.v. injection of a corresponding volume of low molecular weight dextran solution (Rheomacrodex, Pharmacia).

## Results

### *Control values*

Under control conditions in 9 animals, the hepatic arterial mean perfusion pressure (PP) was 115.3 ± 6.0 mmHg and the hepatic arterial blood flow (HABF) 195.2 ± 26.0 ml/min, giving a calculated hepatic arterial vascular resistance (HAVR) of 0.68 ± 0.09 mmHg ml$^{-1}$ min. The livers weighed 274.6 ± 43.5 (s.d.) grams, representing 2.23 ± 0.17 (s.d.) % of the weights of the dogs: expressed per 100 g, the HABF was 71.3 ± 9.5 ml min$^{-1}$ 100 g$^{-1}$ and the HAVR 1.83 ± 0.23 mmHg ml$^{-1}$ min 100 g. The heart rate was 180.4 ± 13.4 beats/min. These values are similar to those reported previously for similar preparations (Richardson & Withrington, 1976b,c).

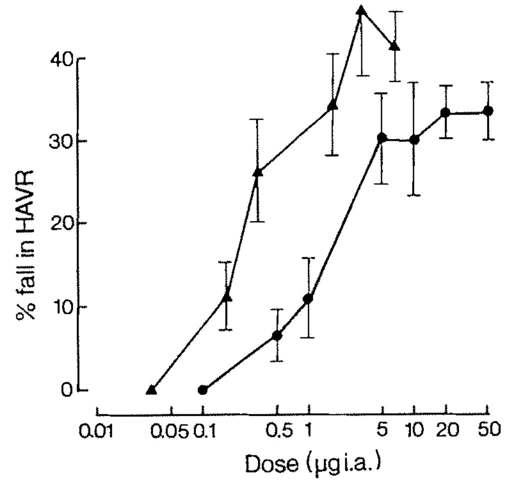

**Figure 1** Log$_{10}$-dose/response curves for pentagastrin (circles) and pancreozymin (triangles). The doses (abscissa) are expressed in terms of the weight of the substances injected i.a. to the liver, and the responses (ordinate) are expressed as the percentage reduction in the calculated hepatic arterial vascular resistance (HAVR). The symbols represent the means of 4 (pancreozymin) or 5 (pentagastrin) injections; the vertical bars represent the s.e. means.

### *Intra-arterial injections of pancreozymin and pentagastrin*

*Pancreozymin.* Pancreozymin was injected in increasing doses between 100 mu and 20 u on one occasion in each of 4 preparations; dose-dependent vasodilatation was the only effect observed above the threshold, which was 500 mu in each experiment (Figures 1 and 2). The maximum reduction in calculated HAVR of 47.9 ± 7.2% was attained on injection of either 10 or 20 u i.a. to the liver (Table 1). The effects of pancreozymin were rapid in onset, and of shorter duration than glucagon (Table 2).

*Pentagastrin.* Pentagastrin was injected i.a. to the liver in graded doses between 0.1 and 50.0 μg on one occasion in each of 5 preparations. Dose-dependent vasodilatation of slow onset was the only effect seen at doses above the threshold of 0.5 μg (Table 2). The

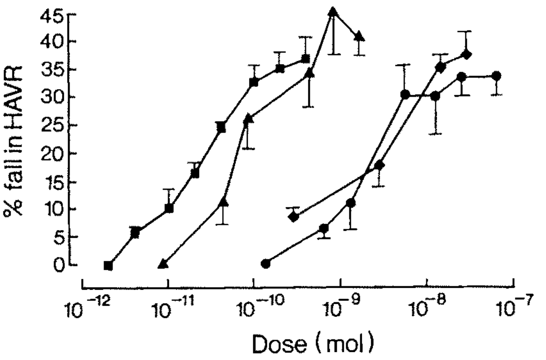

**Figure 2** $Log_{10}$-dose/response curves for glucagon (diamonds), secretin (squares), pancreozymin (triangles) and pentagastrin (circles) injected into the hepatic artery of the anaesthetized dog. The doses are expressed as a fraction of one mole of each substance injected, and the responses are expressed as percentage reductions in calculated hepatic arterial vascular resistance (HAVR). The symbols represent the mean of 4 (glucagon and pancreozymin), 3 (secretin) or 5 (pentagastrin) injections in different experiments, and the vertical bars show the s.e. means.

maximum reduction in calculated HAVR was attained on injection of between 5 and 50 µg in different preparations; at these maximal doses, the HAVR fell by 35.2 ± 3.5%. The changes in PP, HABF and HAVR at maximal vasodilatation are shown in Table 1, and the dose/response relationship summarized in Figures 1 and 2.

### *Relative vasodilator potency of glucagon, secretin, pancreozymin and pentagastrin*

The $\log_{10}$dose/response curves for pancreozymin and pentagastrin, with the doses expressed as a fraction of one mole of the substances are shown in Figure 2, and the $ED_{50}$ values and time courses of the recoveries from selected doses of the agents summarized in Table 2. In addition, the effects of glucagon and secretin, which have been reported previously (Richardson & Withrington, 1976b) are included for comparative purposes.

These results show that secretin and pancreozymin are very much more potent vasodilators of the canine hepatic arterial vascular bed than glucagon or pentagastrin, when administered by i.a. injection.

### *Intra-arterial infusions of glucagon, secretin, pancreozymin and pentagastrin*

All four substances were infused into the hepatic artery in varying doses, to observe whether the resulting vasodilatation was maintained throughout the infusion period or prolonged beyond the cessation of the infusions. The resulting hepatic arterial blood concentrations were calculated by dividing the infusion rate (µg/min or u/min) by the HABF (ml/min), and these calculated blood concentrations are compared with peripheral blood concentrations of the hormones reported to occur physiologically (see Discussion section).

*Glucagon.* Glucagon was infused into the hepatic artery at rates of 0.5, 1.0, 5.0 and 10.0 µg/min (Table 3). At each dose level, there was a sustained decrease in the calculated HAVR due to a substantial increase in HABF with very small reductions in perfusion pressure. The reductions in HAVR are related to both the infusion rate, and the resulting blood concentrations (Table 3, Figures 4 and 5).

The reduction in HAVR on infusion of glucagon was rapid in onset and of very protracted duration, extending beyond the cessation of the infusions by several min, in contrast to the effects of the other substances (Figure 3).

**Table 1** Hepatic arterial perfusion pressure (PP), blood flow (HABF) and calculated hepatic arterial vascular resistance (HAVR) immediately before, and at the peak of responses to maximal vasodilator doses of pancreozymin and pentagastrin

| | *PP* | *PP* | *HABF* | *HABF* | *HAVR* | *HAVR* |
|---|---|---|---|---|---|---|
| | *(mmHg)* | | *(ml min⁻¹ 100 g⁻¹)* | | *(mmHg ml⁻¹ min 100 g)* | |
| | *Control* | *Peak* | *Control* | *Peak* | *Control* | *Peak* |
| Pancreozymin | 93.3 ± 6.1 | 82.3 ± 6.3 | 56.9 ± 15.3 | 91.0 ± 14.7 | 2.09 ± 0.63 | 0.97 ± 0.15 |
| Pentagastrin | 100.2 ± 2.9 | 91.8 ± 3.7 | 71.3 ± 9.3 | 98.6 ± 11.6 | 1.54 ± 0.27 | 1.00 ± 0.14 |

Each value is the mean ± s.e. mean immediately before (Control) and at the peak of the responses to maximal vasodilator doses of the two substances. The data were derived from 4 experiments for pancreozymin and 5 for pentagastrin.

*Secretin.* Secretin was infused i.a. to the liver in doses of 0.5, 1.0 and 5.0 u/min (Table 5). At all doses, there was vasodilatation which was maintained throughout the period of the infusion, and which receded on cessation of the infusions (Figure 3). Even the lowest infusion of secretin, which produced a calculated hepatic arterial blood concentration of 124 pg/ml caused a reduction in HAVR of 2.3%; when 1 u/min was infused, there was a resulting hepatic arterial blood concentration of 382 pg/ml and a statistically significant ($P < 0.005$) reduction in HAVR of 11.3 ± 1.4% (Table 3).

*Pancreozymin.* Pancreozymin was infused at doses of 0.2, 1.0 and 5.0 u/min to the liver on a total of 5 occasions (Table 3). These infusions resulted in calculated hepatic arterial blood concentrations of between 350 and 9260 pg/ml, and all caused reductions

**Table 2** Maximum reductions in hepatic arterial vascular resistance (HAVR), $ED_{50}$ values and times to half recovery from the peak effects of selected doses of each agent ($T_{\frac{1}{2}}$)

| *Substance* | *Maximum fall in HAVR (%)* | *$ED_{50}$ (ng)* | *$ED_{50}$ (mol)* | *Test dose (µg)* | *$T_{\frac{1}{2}}$ to recovery (s)* |
|---|---|---|---|---|---|
| a. Glucagon (*n*=4) | 37.1 ± 4.3 | 1120 | $3.21 \times 10^{-9}$ | 100 | 219.0 ± 16.3 |
| b. Secretin (*n*=3) | 37.1 ± 4.3 | 80 | $2.62 \times 10^{-11}$ | 1.25 | 19.8 ± 3.1 |
| c. Pancreozymin (*n*=4) | 47.9 ± 7.2 | 297 | $7.63 \times 10^{-11}$ | 1.67 | 25.3 ± 10.3 |
| d. Pentagastrin (*n*=5) | 35.2 ± 3.0 | 2500 | $3.25 \times 10^{-9}$ | 20 | 36.4 ± 2.5 |

All effects are shown as means ± s.e. means. The number of experiments from which the data were derived is shown in parentheses after the name of each substance. The data in sections a and b are from Richardson & Withrington (1976b).

**Table 3** The effects of i.a. infusions of glucagon, secretin, pancreozymin and pentagastrin on hepatic arterial vascular resistance

| *Substance* | *Infusion* | *Number of Infusions* | *Blood concentration (ng/ml)* | *Reduction in HAVR (%)* |
|---|---|---|---|---|
| a. Glucagon | 0.5 µg/min | 1 | 1.66 | 3.2 |
| | 1 µg/min | 4 | 6.00 ± 1.80 | 13.4 ± 4.4* |
| | 5 µg/min | 3 | 31.6 ± 9.0 | 28.0 ± 8.9* |
| | 10 µg/min | 5 | 59.5 ± 11.6 | 38.3 ± 7.4*** |
| b. Secretin | 0.5 u/min | 1 | 0.12 | 2.3 |
| | 1 u/min | 4 | 0.38 ± 0.10 | 11.3 ± 1.4*** |
| | 5 u/min | 4 | 1.74 ± 0.24 | 34.0 ± 7.0** |
| c. Pancreozymin | 0.2 u/min | 2 | 0.50 (0.65, 0.35) | 7.6 (7.4, 7.8) |
| | 1 u/min | 2 | 1.99 (1.79, 2.18) | 18.9 (6.4, 31.3) |
| | 5 u/min | 1 | 9.26 | 57.7 |
| d. Pentagastrin | 1 µg/min | 1 | 5.11 | 7.1 (1.8 ƒ) |
| | 5 µg/min | 1 | 33.9 | 64.9 (9.6 ƒ) |
| | 10 µg/min | 2 | 48.1 (61.2, 34.9) | 34.9 (41.9, 27.9)<br>(9.1 ƒ (11.5, 6.7) ƒ) |

Results are shown as means ± s.e. means for 3 or more infusions; for 2 infusions the mean and individual results are given and for 1 infusion, the single result is given. Because of the lack of maintenance of the effects of pentagastrin (see text), the initial and final (ƒ) effects are shown.
*=$P < 0.05$; **=$P < 0.01$; ***=$P < 0.005$.

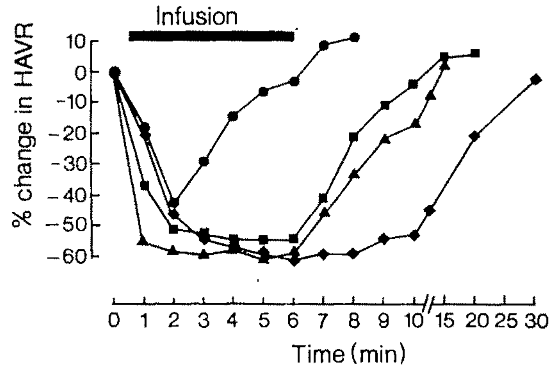

**Figure 3** Time courses of the responses of the hepatic arterial vascular bed to i.a. infusions of glucagon (diamonds, 10 μg/min), secretin (squares, 5 u/min), pancreozymin (triangles, 5 u/min) and pentagastrin (circles, 10 μg/min). Abscissa scale: time in min, the period of the infusions is shown by the horizontal bars. Ordinate scale: % reduction in calculated hepatic arterial vascular resistance (HAVR).

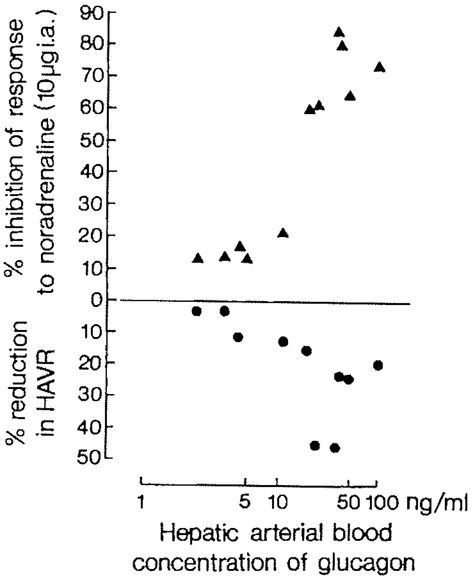

**Figure 4** Relationship between calculated hepatic arterial blood concentration of glucagon resulting from i.a. infusions (abscissa scale) and (i) reduction in calculated hepatic arterial vascular resistance (HAVR, lower section) and (ii) % reduction in the vasoconstrictor response to i.a. injections of noradrenaline (upper section). Each point represents one glucagon infusion.

in calculated HAVR which were dose-dependent and maintained for the duration of the infusions (Figure 3).

*Pentagastrin.* Pentagastrin was infused into the hepatic artery in doses of 1.0, 5.0 and 10.0 μg/min on a total of 4 occasions. Dose-dependent reductions in HAVR occurred with each infusion (Table 3). In contrast to the effects of glucagon, secretin and pancreozymin, however, the hepatic arterial vasodilatation evoked by pentagastrin was not maintained, despite the continued infusions; the HAVR returned towards control levels during all infusions of pentagastrin (Figure 3), and in none of the 4 infusions did the reduction in HAVR at the end of the infusion exceed one third of the reduction attained at the beginning of the infusions (Table 3).

In summary, i.a. infusions of glucagon, secretin, pancreozymin and pentagastrin all evoked reductions in HAVR. The reductions due to secretin and pancreozymin were swift in onset and receded promptly on cessation of the infusions; the effects of glucagon extended beyond the end of the infusion periods, whilst the effects of pentagastrin were evanescent, receding despite the continued infusions.

### *Influence of intra-arterial infusions of glucagon, secretin, pancreozymin and pentagastrin on the responses to intra-arterial injections of noradrenaline*

Intra-arterial injections and infusions of glucagon have previously been shown to antagonize the vasoconstrictor effects of i.a. injected noradrenaline, angiotensin, vasopressin (Richardson & Withrington, 1976a) and adrenaline and 5-hydroxytryptamine (Richardson & Withrington, unpublished observations), but not to antagonize the effects of i.a. administered vasodilator agents (Richardson & Withrington, 1976d). The present investigation was undertaken to examine the relationship between the hepatic arterial blood concentration of glucagon and the extent of the inhibition of the vasoconstrictor effects of i.a. injections of noradrenaline; and also to examine the likelihood of this antagonistic action of glucagon being peculiar to that hormone, or being an effect shared by the other vasodilator hormones secretin and pancreozymin, and by pentagastrin.

*Vasoconstrictor effects of noradrenaline.* The test dose of noradrenaline (10 μg) was injected i.a. to the liver on 124 occasions under control conditions in 35 groups of injections. These injections caused a mean increase in calculated HAVR of 147.5 ± 7.7% ($n = 35$), a value similar to that previously reported for the injection of this dose of noradrenaline into the sympathetically-denervated hepatic arterial vascular bed of the dog (Richardson & Withrington, 1967a).

*Effect of glucagon on the response to noradrenaline.* The relationship between the administered dose and the blood concentration of glucagon and (i) the reduction in HAVR and (ii) the percentage inhibition of the response to i.a. injections of noradrenaline is illustrated in Figures 4 and 5. The effects of the various infusions of glucagon are shown in Table 4: even the lowest infusion of glucagon which resulted in a calculated hepatic arterial blood concentration of 1.66 ng/ml caused inhibition of the response of the hepatic arterial vascular bed to noradrenaline; this inhibition was statistically significant when 1 µg/min was infused to 3 preparations, resulting in hepatic arterial blood concentrations of 5.8 ± 2.0 ng/ml. The inhibition of the vasoconstrictor responses to noradrenaline occurred over the same range of hepatic arterial blood glucagon concentrations as the reductions in HAVR (Figures 4 and 5).

*Effect of secretin on the response to noradrenaline.* Intra-arterial infusions of secretin, 1.0 and 5.0 u/min, which produced graded reductions in HAVR did not antagonize the effects of i.a. injected noradrenaline. Indeed, the vasoconstrictor effects of noradrenaline were apparently potentiated (Figure 5, Table 4). This effect is probably not a genuine potentiation since the noradrenaline was acting upon a more relaxed hepatic arterial vasculature during the secretin infusion than under control conditions. When 1.0 u/min of secretin was infused i.a., the resulting hepatic arterial blood concentration was 380.0 ± 100.4 pg/ml ($n=4$) and the response to noradrenaline 4.5 ± 4.3% greater than during control conditions.

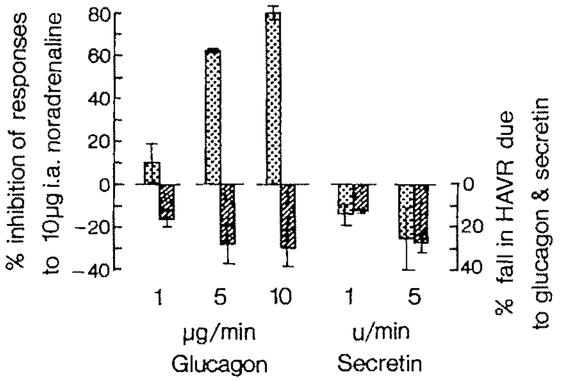

**Figure 5** Differences in the response of the hepatic arterial vasculature to i.a. infusions of glucagon and secretin. Hatched bars show the % reduction in calculated hepatic arterial vascular resistance (HAVR) due to the infusions; stippled bars show the influence of the infusions on the response of the hepatic arterial vasculature to i.a. injections of noradrenaline (10 µg). Stippled bars above the baseline indicate that the effect of noradrenaline during the infusion was smaller than that during corresponding control periods. Bars represent the means of 3 infusions at each dose level and the lines within the main bars show the s.e. means

*Effect of pancreozymin on the response to noradrenaline.* Intra-arterial infusions of pancreozymin were made at two dose levels to examine their effects on the vasoconstrictor responses of this vascular bed to noradrenaline. Two infusions of 200 mu/min resulted in a mean calculated hepatic arterial blood concentration of 499 pg/ml and an apparent potentiation of the vasoconstrictor response to noradrenaline of 6.4%; two infusions of 1 u/min resulted in a mean blood concentration of 1985 pg/ml

**Table 4** The effects of i.a. infusions of glucagon and secretin on the responses of the hepatic arterial vasculature to i.a. injections of noradrenaline

| *Substance* | *Infusion rate* | *Number of observations* | *Change in response to noradrenaline during infusion compared to the response under control conditions* |
|---|---|---|---|
| a. Glucagon | 0.5 µg/min | 1 | −13.0% |
| | 1 µg/min | 3 | −12.9 ± 3.4%* |
| | 5 µg/min | 3 | −45.5 ± 11.1%* |
| | 10 µg/min | 3 | −78.7 ± 5.3%** |
| b. Secretin | 1.0 u/min | 4 | + 4.5 ± 4.3% |
| | 5.0 u/min | 3 | +23.4 ± 11.7% |

Values are shown as means ± s.e. means except for individual values; * $=P<0.05$; ** $=P<0.0025$; unmarked values represent changes which are not statically significant ($P>0.05$).

and an apparent potentiation of the effect of noradrenaline of 11.9%. The pattern of the effects of pancreozymin on the vasoconstrictor responses of the hepatic arterial vasculature to noradrenaline is therefore very similar to that for secretin (Figure 5).

*Effect of pentagastrin on the response to noradrenaline.* Pentagastrin was infused into the hepatic artery in doses of 1, 5 and 10 µg/min and these infusions resulted in hepatic arterial blood concentrations of 5.11, 33.9 and 34.9 ng/ml respectively. When the hepatic arterial vasculature was dilated by pentagastrin (Figure 3), the vasoconstrictor responses to i.a. injections of noradrenaline in these three experiments were potentiated by 1.3, 7.3 and 16.6% respectively. Quantitative results were difficult to obtain with pentagastrin because of the very marked tendency of the direct vasodilator effect to wane despite the continued infusions; the apparent potentiation of the response to noradrenaline was only clear when the hepatic arterial vasculature was demonstrably dilated by the agent.

In summary, the antagonism of the vasoconstrictor effects of test doses of noradrenaline on the hepatic arterial vascular bed of the dog is peculiar to glucagon of the four agents examined. This antagonism by glucagon of the vasoconstrictor effect of noradrenaline has been shown to be dependent upon the hepatic arterial blood concentration (Figure 4) and infused dose (Figure 3) of glucagon.

## Discussion

In previous experiments it has been shown that hepatic arterial injections or infusions of glucagon inhibit the hepatic arterial vasoconstriction produced by noradrenaline, angiotensin and vasopressin (Richardson & Withrington, 1976a) and adrenaline (Richardson & Withrington, unpublished). Furthermore the interaction of glucagon with noradrenaline or angiotensin is competitive in nature whilst that between glucagon and vasopressin is noncompetitive. In the present series of experiments the dose range over which glucagon evokes hepatic arterial vasodilatation and inhibition of hepatic arterial vasoconstriction by noradrenaline has been examined down to threshold concentrations. There was a clear correlation between the two effects and they were not separable even at the lowest infusion rates of glucagon. The lowest arterial infusion of glucagon in the present experiments produced a calculated arterial blood concentration of 1.66 ng/ml and the test dose of arterial noradrenaline was inhibited by 13%; a calculated arterial concentration of 60.0 ng/ml glucagon caused an inhibition of over 75% in the arterial vasoconstriction to noradrenaline. All arterial concentrations of glucagon which antagonized the hepatic arterial vasoconstrictor effects of noradrenaline also evoked profound vasodilatation of the hepatic arterial bed (Figure 4).

The peripheral plasma concentration of glucagon has been measured by radioimmunoassay (RIA); in man and in monkeys, the normal fasting levels of glucagon in peripheral plasma fall within the range of 25–300 pg/ml (Rehfeld & Heding, 1970; Bloom, Daniel, Johnston, Ogawa & Pratt, 1973; Dudl, Lerner, Ensinck & Williams, 1973; Weir, Turner & Martin, 1973; Tasaka, Sekine, Wakatsuki, Ohgawara & Shizume, 1975) and in dogs, a resting plasma concentration of glucagon in the inferior vena cava of 101 ± 25 pg/ml ($n = 4$) has been reported (Santeusanio, Faloona & Unger, 1973). There is, therefore, a clear discrepancy between the plasma levels of glucagon as measured by RIA which occur under physiological conditions, and the hepatic arterial blood concentrations which were calculated in the present experiments (i.e. 1–100 ng/ml) and which produce vasodilatation in the hepatic arterial vascular bed, concomitantly with inhibition of the vasoconstrictor effects of noradrenaline. The disparity between these levels may be due to a number of factors: the calculated concentrations do not take into account either the destruction of the hormone in the blood, or its deactivation or binding to a biologically inert form. A further possibility is that the part of the molecule of glucagon to which an antibody has been raised for purposes of the RIA estimation is not identical with that moiety responsible for the effects observed in the present experiments of hepatic vasodilatation and inhibition of hepatic vasoconstriction evoked by noradrenaline.

The prolonged time-course of action of injections of glucagon on the hepatic arterial bed has been commented on previously (Richardson & Withrington, 1976b); the present experiments reveal the same feature to be present after intra-arterial infusions of the hormone (Figure 3). The cumulative action inherent in this property means that considerably smaller amounts of the hormone may be present under physiological conditions and may cause a significant hepatic vasodilatation. In addition, other substances may be present, like secretin, pancreozymin and prostaglandins; these have profound hepatic arterial vasodilator properties themselves (Richardson & Withrington, 1976b) which are not antagonized by glucagon (Richardson & Withrington, 1976d).

Secretin has been shown to be a vasodilator of various peripheral vascular beds in the cat (Ross, 1970) and, in the dog, to be as potent as prostaglandin $E_2$ in evoking vasodilatation of the hepatic arterial vascular bed (Richardson & Withrington, 1976b) when administered into the hepatic artery by injection. In the present series, infusions of secretin were given i.a. in doses which, on calculation, produced blood concentrations of 125–2000 pg/ml. Vasodilatation was observed at all doses and was maintained throughout the period of infusion.

In man, fasting secretin levels range between about 50 and 500 pg/ml of plasma (Bloom & Ogawa, 1973; Boden & Chey, 1973; Bloom, 1974; Henry, Flanagan & Buchanan, 1975). Feeding and the instillation of acid into the duodenum cause these levels to rise in man (Boden & Chey, 1973; Bloom & Ogawa, 1973; Henry *et al.*, 1975) and so it is not unreasonable to assume that if the haematocrit is about 50%, blood concentrations of 25–300 pg/ml of secretin fall within the physiological range. The two lower levels of infusion used in the present series would produce blood concentrations within this range. Therefore significant reductions in HAVR may be expected to accompany the physiological secretion of secretin. In contrast to the hepatic arterial infusions of glucagon, which significantly inhibit the hepatic vasoconstrictor responses to i.a. injections of noradrenaline, arterial infusions of secretin, whilst evoking comparable reductions in HAVR, increase the vasoconstrictor actions of noradrenaline. However, our experiments suggest that this increased responsiveness of the vessels to noradrenaline arises from a reduction in the background hepatic arterial vascular tone induced by the secretin infusion rather than a potentiation of noradrenaline by the secretin.

Pancreozymin was investigated for the first time in the present experiments. Injections into the hepatic artery produced dose-dependent vasodilatation of brief duration; its potency was only slightly less than secretin (Table 2). Infusions into the hepatic artery evoked concentration-dependent hepatic arterial vasodilatation which was maintained throughout the period of infusion and very quickly subsided after cessation of the infusion. Vasoconstrictor responses to i.a. injections of test doses of noradrenaline were increased during the infusion of pancreozymin, in a similar manner to that which occured during the infusions of secretin. Similarly, this increase in response is considered secondary to the vascular smooth muscle relaxing properties which caused the reduction in HAVR. Peripheral serum concentrations of pancreozymin in man have been reported to range between 25–60 pg/ml (Harvey, Dowsett, Hartog & Read, 1973; 1974) and as high as 4 ng/ml (Reeder, Becker, Smith, Rayford & Thompson, 1973). These levels rise to between 9 and 17 ng/ml after food containing fats (Harvey *et al.*, 1973; Reeder *et al.*, 1973). The calculated hepatic arterial blood concentrations in the present series of experiments (0.35–9.26 ng/ml) therefore fall well within the range of peripheral pancreozymin levels determined by RIA in man after meals. It therefore seems probable that significant reductions in HAVR are produced by pancreozymin released in response to physiological stimuli.

The various molecular fractions of gastrin which have been reported are not available in sufficient quantity to be used in this type of experiment. The physiological action of gastrin as a gastric secretogogue resides in the 5 amino acid sequence which is available as the commercial preparation pentagastrin. In the present experiments injections of pentagastrin evoked dose-dependent vasodilatation of the hepatic arterial bed of longer duration than either secretin or pancreozymin; its molar potency was less than either of these and close to that of glucagon (Table 2, Figure 2). In contrast to the three naturally-occurring hormones, the hepatic vasodilator activity of pentagastrin on i.a. infusion was not maintained, a reflection, perhaps, of its rapid deactivation by the liver (Thompson, Reeder, Davidson, Charters, Bruckner, Lemmi & Miller, 1969; Temperley, Stagg & Wyllie, 1971). Because of the rapid decline of the vasodilator response to pentagastrin any alteration of the hepatic arterial vasoconstrictor properties of noradrenaline was difficult to ascertain in these experiments. Nevertheless it was apparent that whilst an hepatic arterial vasodilatation was present due to pentagastrin the vasoconstrictor responses to noradrenaline were increased. This potentiation was as evanescent as the vasodilator response to pentagastrin and supports the conclusions drawn from the secretin and pancreozymin infusions that the effect is a secondary one accompanying a primary action relaxing the vascular smooth muscle.

The question arises of the physiological importance of these observations on the vascular effects of commercial preparations of the gastrointestinal hormones. A comparison of the calculated hepatic arterial blood concentrations of secretin and pancreozymin with the plasma or serum levels determined in other investigations indicates that a significant reduction in vascular resistance in the liver occurs due to their presence in the systemic arterial circulation following physiological stimuli which provoke their release, as in the small intestine (Fara, Rubinstein & Sonnenschein, 1972; Richardson, 1976). During digestion, secretin and pancreozymin would be released concomitantly; previous reports have demonstrated that mesenteric vasodilatation occurs after eating in dogs (Fronek & Stahlgren, 1968), and in cats, the release of gastrointestinal hormones, particularly secretin and pancreozymin, has been strongly implicated in the mediation of mesenteric vasodilatation resulting from the intraduodenal instillation of fat and other substances (Fara *et al.*, 1972).

There was, in the present experiments, a marked similarity between the effects, and the molar potencies, of secretin and pancreozymin. This may reflect an essentially similar vascular action of these structurally-related hormones, but in addition, the possibility that impurities in the preparations may have contributed to their vasoactivity arises. Fara (1975) has noted the possible contamination of Boots secretin with pancreozymin, and since on the isolated portal vein at least, secretin and pancreozymin cause mutual potentiation of their effects (Fara, 1975), such contamination of one hormone with the other could have contributed to the apparent potency of these two preparations used in the present experiments.

The present experiments represent strong evidence that preparations of natural secretin and pancreozymin cause hepatic arterial vasodilatation which may well occur at physiological concentrations. The concomitant release of pancreatic glucagon might contribute further to hepatic arterial vasodilatation, and in addition, could protect the hepatic arterial vasculature from the vasoconstrictor effects of circulating adrenaline and angiotensin. Therefore, in addition to their well-established primary roles, the gastrointestinal hormones studied in this investigation may, by increasing HABF, participate in ensuring an adequate supply of oxygen to the liver parenchyma for all the biochemical reactions associated with post-absorptive liver function. This would be particularly important due to the greater requirements of the gastrointestinal tract, and it is at this time that the hepatic arterial blood concentrations of these hormones would be greatest.

This study was supported by the Medical Research Council; we wish to thank the Boots Company (Research Division) for assistance in a literature search and Miss Dorinda Lobendhan and Miss June Pyke for technical assistance.

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*(Received June 11, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 157–162

# EFFECT OF ANTI-THYROID AGENTS, METHIMAZOLE AND PROPYLTHIOURACIL, ON BRAIN NORADRENALINE CONTENT

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1 Methimazole (1-methyl-2-mercaptoimidazole, MMI) and propylthiouracil (6-propyl-2-thiouracil, PTU) which are used in the therapy of hyperthyroidism were found to reduce brain noradrenaline (NA) content. Endogenous NA levels in rat brain were reduced from 1 to 6 h after intraperitoneal injection of MMI by doses in excess of 25 mg/kg and by PTU at a dose of 50 mg/kg. However, endogenous NA in the rat heart was only slightly reduced after 50 mg/kg of MMI, and was not affected by PTU (50 mg/kg).

2 Both MMI and PTU effectively inhibited the *in vivo* conversion of [$^3$H]-dopamine into [$^3$H]-noradrenaline ([$^3$H]-NA) in the brain of rats after a single intraperitoneal injection of doses above 10 mg/kg (MMI) and 25 mg/kg (PTU). This inhibition by MMI and PTU was dose-dependent over the range of 10 mg/kg to 50 mg/kg, was highest after 2–3 h and continued for at least 6 h after their injection. The conversion rates returned to normal after 24 hours.

3 The results suggest that the reduction of brain NA by these drugs is, at least in part, due to the inhibition of brain dopamine $\beta$-hydroxylase.

## Introduction

Methimazole (1-methyl-2-mercaptoimidazole, MMI) and 6-propyl-2-thiouracil (PTU) are potent inhibitors of thyroid iodide peroxidase, which catalyzes the initial step of thyroid hormone biosynthesis (Mahoney & Igo, 1966; Coval & Taurog, 1967) and are used clinically in the therapy of hyperthyroidism.

Various sulphydryl compounds such as mercaptoethanol, cysteine, glutathione and coenzyme A are known to be inhibitors of dopamine $\beta$-hydroxylase [3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (hydroxylating), EC 1.14.2.1.] (DBH) which catalyzes the biosynthesis of noradrenaline (NA) from dopamine (Nagatsu, Kuzuya & Hidaka, 1967). As MMI has a sulphydryl group, and PTU also contains sulphur, studies on the effect of the two drugs on catecholamine metabolism *in vivo* were carried out. This paper reports that MMI and PTU effectively reduce brain NA content probably by inhibiting brain DBH *in vivo*.

## Methods

Male Wistar rats weighing approximately 150 g were kept at a constant temperature in an environment calculated to be stress-free. The animals were exposed for at least ten days to regular light-dark cycles before starting the experiments. Rats were killed by decapitation; the tissues were quickly removed, weighed and stored at −80°C until biochemically analysed. MMI and PTU were suspended in 0.5% carboxymethylcellulose (CMC) and 0.5 ml of these solutions was injected intraperitoneally into rats. Control rats received an intraperitoneal injection of 0.5 ml of 0.5% CMC. Satistical significance was determined by the use of Student's *t* test (Finney, 1967). [$^3$H]-Dopamine (5–10 Ci/mmol) was purchased from New England Nuclear. All other chemicals were reagent grade.

### *Noradrenaline determination*

NA was isolated by alumina adsorption (Anton & Sayre, 1962) and estimated fluorometrically after ferricyanide oxidation of 0.4 ml of the final acid eluate. NA standards were added to aliquots of tissue and processed in the same way as the test samples. All values were corrected for recoveries of 70% ± 10% s.d. Five rats were used per group.

### *Dopamine β-hydroxylase assay* in vitro

DBH of bovine adrenal gland was purified by ammonium sulphate precipitation, charcoal treatment, calcium phosphate gel treatment, and DEAE-cellulose column chromatography as described by Friedman & Kaufman (1965). The activity of bovine adrenal DBH was determined spectrophotometrically using tyramine as the substrate according to Creveling, Daly, Witkop & Udenfriend (1962), and the activity of rat brain DBH was measured radioisotopically according to Molinoff, Weinshilboum & Axelrod (1971). Rat brains were homogenized with glass homogenizers in 10 volumes of cold tris (hydroxymethyl)aminomethane (Tris) buffer, 0.005 M, pH 7.4, containing Triton X-100, 0.1%. Homogenates were centrifuged at 15,000 *g* for 10 min and portions of the supernatants were used for assay of DBH. DBH activity was not detectable in the pellets. The two stage assay for DBH is based on the conversion of tyramine to octopamine by DBH, followed by N-methylation of the β-hydroxylated product with a $^{14}$C-labelled methyl group from S-adenosylmethionine. The second reaction was catalyzed by bovine adrenaal phenylethanolamine *N*-methyltransferase (PNMT), partially purified by the method of Axelrod (1962) by means of ammonium sulphate precipitation and Sephadex G-200 to separate PNMT from DBH. The reaction mixture contained: 200 µl of tissue preparation corresponding to 5 to 50 mg of wet tissue weight, 0.12 µmol of tyramine, and 10 µmol of Tris buffer, pH 6.0. Sufficient catalase (1500 units) and $CuSO_4$ (30 to 80 nmol per tube) were added to give maximal activity.

### *Conversion of [$^3$H]-dopamine to [$^3$H]-noradrenaline in rat brain*

Conversion of [$^3$H]-dopamine to [$^3$H]-NA in rat brain was determined by giving [$^3$H]-dopamine intracisternally as a precursor (Hidaka, Hara, Harada, Hashizume & Yano, 1974). Twenty-five µl of radioisotope solution ([$^3$H]-dopamine, 5 µCi per rat) was given intracisternally; CMC, PTU or MMI were injected (i.p.) at 1, 2, 5, 6 or 24 h before the injection of [$^3$H]-dopamine. Animals were killed 10 min after the administration of [$^3$H]-dopamine. Whole brains were disintegrated with a glass tissue homogenizer in 7.5 ml of 0.4 N perchloric acid solution containing 50 µg of dopamine, NA, normetanephrine, homovanillic acid and 3-methoxytyramine. The tissue extracts were passed over an AG-50 (H$^+$) column and amines were adsorbed to the resin and were thus separated from the acid and neutral catabolites which passed through in the effluent. The method of Carlsson & Waldeck (1963) was used with an 80 by 5 mm column and a modified elution procedure (Hidaka *et al.,* 1974). When the column was eluted with 15 ml of 0.4 N HCl, both acid and neutral catabolites were found in the first 5 ml eluate as well as in the fractions which immediately followed (i.e., fractionated eluates), each containing 5 ml. The adsorbed amines were eluted with 60 ml of 1 N HCl and the following 20 ml of 2 N HCl. When [$^3$H]-dopamine was used as the precursor, NA was collected in fractions 4 to 6 and dopamine was collected in fractions 8 to 12 (each fraction representing 5 ml). A portion (0.25 ml) of each fraction was transferred to scintillation vials containing 10 ml scintillation fluid, Bray's solution (120 g naphthalene, 0.4 g POPOP, 8 g PPO, 200 ml methanol, 40 ml ethylene glycol, 2 l dioxane). Radioactivity was determined in a liquid scintillation spectrometer (Beckman model LS 233). The efficiency (24.2 ± 0.6% tritium) was determined by the use of internal standards. The amounts (nCi) of isolated amines were calculated from determined radioactivity (ct/min) using this efficiency.

### *Assay of noradrenaline release*

The method of Daly, Creveling & Witkop (1966) was employed for this study. Isotonic NaCl solution (0.2 ml) containing 12.5 µCi of [$^3$H]-NA was injected into the tail vein of mice. Drugs were administered intraperitoneally one hour after the radioisotope injection. The mice were killed by a blow on the head 3 h after the radioisotope injection. Hearts were immediately weighed and homogenized in 10 volumes (w/v) of 0.4 N perchloric acid. After centrifugation, 0.25 ml of the supernatant solution was added to 10 ml of Bray's solution, and radioactivity was determined by liquid scintillation counting. At least six mice were used for each group.

## Results

### *Inhibition of dopamine β-hydroxylase* in vitro

Bovine adrenal and rat brain DBH were inhibited by MMI and PTU *in vitro.* The inhibitory effect of these drugs is summarized in Table 1. Unlike the sulphydryl compounds reported previously to inhibit DBH (Nagatsu *et al.,* 1967), the inhibition of DBH by MMI and PTU was not reversed by the addition of N-ethylmaleimide (NEM). These results might suggest that inhibition of DBH by MMI and PTU is not due to simple chelation between these compounds and copper in the enzyme.

### *Effects of methimazole and propylthiouracil on noradrenaline content of rat brain and heart*

In Table 2 the effects of MMI on rat brain and heart NA content are shown at various times after

**Table 1** Effects of methimazole (MMI) and propylthiouracil (PTU) on bovine adrenal and rat brain dopamine β-hydroxylase (DBH) *in vitro*

| | $I_{50}$* *(m)* | | | |
|---|---|---|---|---|
| *Compounds* | *Without NEM* | | *With 10 mM NEM*** | |
| | Adrenal | Brain | Adrenal | Brain |
| MMI | 3.7 | 1.2 | 3.5 | — |
| PTU | 1.5 | 1.8 | 1.8 | — |

* Concentration of drug producing 50% inhibition of DBH activity, as determined graphically. ** N-ethylmaleimide (NEM) was not added to the brain DBH assay system because the compound inhibited phenylethylamine-*N*-methyltransferase (PNMT) activity. PTU and MMI did not affect the activity of PNMT.

**Table 2** Effects of methimazole (i.p.) on noradrenaline content (μg/g) of rat brain and heart

| | *Dose* | *Time post drug (h)* | | | |
|---|---|---|---|---|---|
| *Tissue* | *(mg/kg)* | 1 | 2 | 6 | 24 |
| | | | *Noradrenaline (μg/g)* | | |
| Brain | Control | 0.40 ± 0.02 | 0.41 ± 0.01 | 0.40 ± 0.02 | 0.43 ± 0.03 |
| | 10 | 0.34 ± 0.01 | 0.36 ± 0.02 | 0.37 ± 0.01 | 0.40 ± 0.01 |
| | 25 | 0.30 ± 0.01*,** | 0.31 ± 0.01*,** | 0.35 ± 0.01 | 0.44 ± 0.02 |
| | 50 | 0.27 ± 0.01*,** | 0.27 ± 0.01*,** | 0.30 ± 0.01*,** | 0.43 ± 0.02 |
| Heart | Control | 1.10 ± 0.07 | 1.05 ± 0.09 | 1.11 ± 0.08 | 1.24 ± 0.12 |
| | 10 | 0.92 ± 0.05 | 1.00 ± 0.05 | 1.14 ± 0.09 | 1.13 ± 0.03 |
| | 25 | 0.96 ± 0.07 | 0.80 ± 0.06 | 0.97 ± 0.05 | 1.04 ± 0.05 |
| | 50 | 0.79 ± 0.05* | 0.89 ± 0.11 | 0.93 ± 0.05 | 0.89 ± 0.05 |

Five rats were used for each experiment and values are shown as mean ± s.e.
* Significantly different from control ($P < 0.05$); ** Significantly different from the value at 24 h ($P < 0.05$).

**Table 3** Effects of propylthiouracil i.p. on noradrenaline content (μg/g) of rat brain and heart

| | *Dose* | | *Time post drug (h)* | |
|---|---|---|---|---|
| *Tissue* | *(mg/kg)* | 2 | 6 | 24 |
| | | | *Noradrenaline (μg/g)* | |
| Brain | Control | 0.46 ± 0.01 | 0.47 ± 0.01 | 0.44 ± 0.01 |
| | 25 | 0.42 ± 0.01 | 0.44 ± 0.01 | 0.43 ± 0.02 |
| | 50 | 0.35 ± 0.01*,** | 0.38 ± 0.01* | 0.43 ± 0.01 |
| Heart | Control | 0.96 ± 0.05 | 0.99 ± 0.09 | 0.97 ± 0.04 |
| | 25 | 0.89 ± 0.04 | 0.99 ± 0.05 | 0.98 ± 0.06 |
| | 50 | 0.99 ± 0.03 | 0.97 ± 0.08 | 0.96 ± 0.05 |

Five rats were used for each experiment and values are shown as mean ± s.e.
* Significantly different from control ($P < 0.05$); ** Significantly different from the value at 24 h ($P < 0.05$).

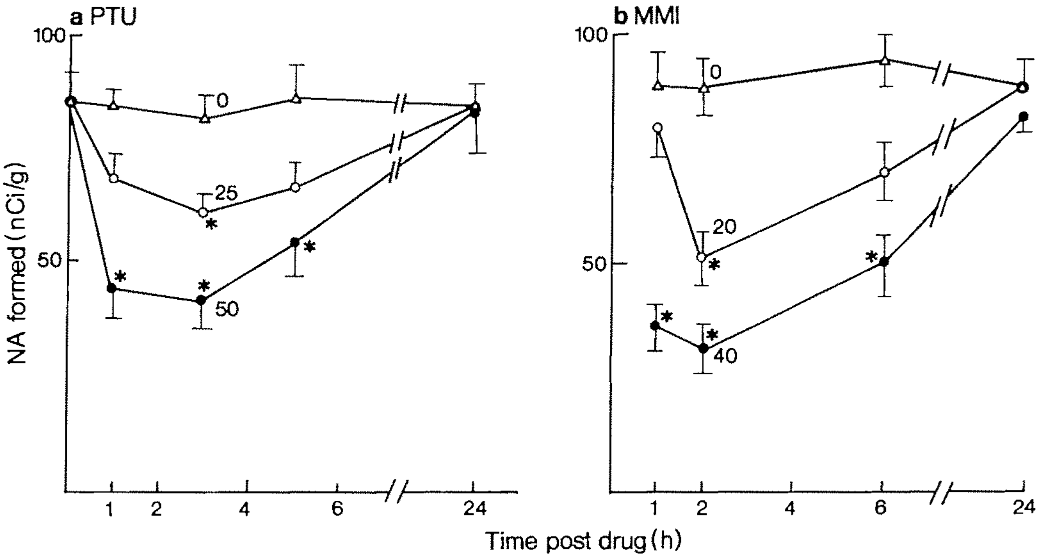

Figure 1 Effects of methimazole (MMI) and propylthiouracil (PTU) on the conversion of [$^3$H]-dopamine into [$^3$H]-noradrenaline ([$^3$H]-NA) in rat brain as a function of time. Five rats were used for each experiment. Drugs were injected intraperitoneally; 5 µCi of [$^3$H]-dopamine per rat (25 µl) was injected intracisternally at the time indicated after drug injection. The brain was removed 10 min after [$^3$H]-dopamine injection. NA was isolated as described in the Methods section. Indicated values (nCi) were calculated from ct/min of NA isolated (efficiency 24%). Five determinations were averaged. Vertical bars indicated standard errors. (△) Control; (a) PTU: (○) 25 and (●) 50 mg/kg; (b) MMI (○) 20 and (●) 40 mg/kg. * Significantly different from the control and the value at 24 h ($P<0.05$).

intraperitoneal injection of different doses of MMI. Brain NA was significantly reduced at doses of 25 and 50 mg/kg. Maximum reduction of brain NA occurred 1–2 h after the drug was given. Heart NA decreased significantly 1 h after MMI only at a dose of 50 mg/kg and the effect of MMI on heart NA was significantly weaker than that of the drug on brain NA. Table 3 summarizes the effects of PTU on NA in rat brain and heart. Maximum reduction of brain NA was observed 2 h after this drug. Heart NA did not decrease significantly. PTU proved to be more toxic than MMI, the intraperitoneal injection of more than 100 mg/kg of PTU killing most of the animals. Accordingly experiments with PTU were performed using doses of

Table 4 Effects of methimazole and propylthiouracil on the release of [$^3$H]-noradrenaline ([$^3$H]-NA) from mouse heart

| *Drug* | *Dose (mg/kg)* | *No. of animals* | ct $min^{-1}$ $g^{-1}$ × $10^{-4}$ |
|---|---|---|---|
| Methimazole | 25 | 7 | 22.1 ± 1.4 |
| | 50 | 7 | 19.2 ± 1.0 |
| | 100 | 7 | 17.6 ± 2.0 |
| Polythiouracil | 25 | 7 | 16.8 ± 2.6 |
| | 50 | 7 | 17.1 ± 2.2 |
| Reserpine | 1 | 6 | 4.1 ± 0.3* |
| | 5 | 6 | 1.8 ± 0.2* |
| Control (saline) | | 10 | 20.8 ± 0.9 |

[$^3$H]-NA (12.5 µCi) was injected into the tail vein of mice. Drugs were administered (i.p.) 1 h after radioisotope injection. The mice were killed 3 h after the radioisotope injection. The hearts were homogenized with 10 volumes of 0.4 N perchloric acid. Values are presented as ct $min^{-1}$ $g^{-1}$ ± s.e.
* Significantly different from control ($P<0.01$)

**Table 5** Effect of methimazole on the conversion of intracisternally injected [$^3$H]-dopamine into [$^3$H]-noradrenaline ([$^3$H]-NA) in rat brain

| Drug | Dose (mg/kg) | No. of animals | Total radioactivity<br>% injected dose | <br>nCi/g | [$^3$H]-NA nCi/g | [$^3$H]-dopamine nCi/g |
|---|---|---|---|---|---|---|
| Control | | 10 | 8.0 | 399 ± 3.9 | 88.7 ± 7.0 | 181 ± 11 |
| Methimazole | 10 | 5 | 8.0 | 400 ± 4.4 | 50.4 ± 5.7* | 239 ± 15 |
| | 40 | 5 | 7.7 | 384 ± 15 | 32.9 ± 4.2* | 232 ± 11 |
| | 100 | 5 | 7.6 | 378 ± 18 | 27.7 ± 3.5** | 231 ± 12 |
| | 250 | 5 | 8.2 | 408 ± 17 | 26.0 ± 3.1** | 214 ± 10 |

Drug was administered (i.p.) to rats 2 h before intracisternal injection of [$^3$H]-dopamine (10 µCi/rat). Rats were killed by decapitation 10 min after [$^3$H]-dopamine and brains were removed. [$^3$H]-NA and [$^3$H]-dopamine were isolated as described in the Methods section. Values are expressed as mean ± s.e. and calculated from ct/min of [$^3$H]-dopamine or NA (efficiency: 24%).

50 mg/kg or less. None of the rats treated with an intraperitoneal injection of 25 or 50 mg/kg of PTU died, at least, not during the first 24 hours. Effects of MMI and PTU on NA release from mouse heart were also examined. Neither MMI nor PTU caused a significant release of NA at doses of 25, 50 or 100 mg/kg. Under the same conditions reserpine was found to release NA (Table 4).

### *Effects of methimazole and propylthiouracil on the* in vivo *conversion of [$^3$H]-dopamine into [$^3$H]-noradrenaline*

As shown in Table 5, the reduced formation of [$^3$H]-NA from [$^3$H]-dopamine in rat brain by MMI was dose-dependent over the range of 10–40 mg/kg. *In vivo* inhibition of DBH by MMI and PTU in rat brain was studied by following the time course of the reduction of [$^3$H]-NA formation at various times after drug administration (Figure 1). The biosynthesis of NA from dopamine decreased rapidly, and maximum inhibition of NA biosynthesis in the brain was attained after 2 hours. This reduced biosynthesis of NA had returned to control levels after 24 hours.

## Discussion

MMI and PTU are used clinically in the therapy of hyperthyroidism and are also known to be inhibitors of thyroid iodide peroxidase. Both MMI and PTU inhibit DBH *in vitro*. The concentrations of MMI and PTU producing 50% inhibition of adrenal DBH *in vitro* were 3.7 and 1.5 mM respectively. Hidaka, Asano & Takemoto (1973) have reported that fusaric acid and its derivatives produce 50% inhibition of DBH *in vitro* at a concentration of 0.1 µM. In spite of the relatively weak *in vitro* inhibitory activity of MMI and PTU, they significantly inhibited *in vivo* conversion of [$^3$H]-dopamine to [$^3$H]-NA in doses similar to those for fusaric acid and its derivatives (Hidaka *et al.*, 1974). The *in vivo* inhibition of the conversion of dopamine to NA by MMI and PTU indicates that they inhibited DBH. Intraperitoneal injection of MMI (above 25 mg/kg) and PTU (50 mg/kg) significantly reduced the brain noradrenaline content *in vivo*. However, the results shown in Table 2 indicate that they do not cause this reduction by virtue of a reserpine-like effect. There is a possibility that the decreased biosynthesis of thyroid hormone by the compounds indirectly affects brain NA content, because there are reports that myocardial NA is altered by both hypothyroidism and hyperthyroidism (Goodall, 1951; Goodkind, Farm & Roberts, 1961; Gordon, Reid, Sjoerdsma & Udenfriend, 1966). However, this possibility is unlikely because PTU did not affect myocardial NA and MMI also had a much weaker effect on the myocardium than it did on the brain. This leaves the conclusion that the effect in high doses is exerted by their action on DBH but the problem of why brain NA is affected so very much more than heart NA remains.

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*(Received June 16, 1976.*
*Revised August 29, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 163–175

# INTERACTIONS BETWEEN THE EFFECTS OF α- AND β-ADRENOCEPTOR AGONISTS AND ADENINE NUCLEOTIDES ON THE MEMBRANE POTENTIAL OF CELLS IN GUINEA-PIG LIVER SLICES

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1 The β-adrenoceptor agonist isoprenaline normally causes only a small and inconsistent increase in the membrane potential of cells in guinea-pig liver slices, in contrast to the large hyperpolarizations seen with α-agonists. However, after a selective α-adrenoceptor agonist has been applied, the response to isoprenaline becomes greatly enhanced.

2 Simultaneous application of small doses of an α- and a β-agonist produce hyperpolarizations larger than the sum of the responses to each agent alone.

3 These interactions occur with a range of sympathomimetic amines, including some which are not substrates for various processes for the uptake and inactivation of catecholamines.

4 Hyperpolarizations caused by externally applied cyclic adenosine-3′,5′-monophosphate (cyclic AMP) also become larger after application of an α-agonist.

5 The adenine nucleotides adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP) hyperpolarize guinea-pig liver cells in the dose range 0.1–1.0 mM. This response is not increased after an α-agonist. However, ADP and ATP are themselves able to enhance the response to β-agonists.

6 These interactions between α-agonists, β-agonists and adenine nucleotides seem to involve steps subsequent to receptor activation. Changes in the intracellular actions of cyclic AMP may be concerned.

## Introduction

In addition to their well-established effects on glucose release, adrenaline and noradrenaline *in vivo* cause a net loss of potassium from the livers of several species. Both actions can be demonstrated *in vitro* using liver slices which thus provide a convenient preparation for the study of the underlying mechanisms. Experiments with guinea-pig liver slices have shown that the potassium loss is accompanied by an increase in membrane potential and a fall in membrane resistance, and can be explained as a consequence of an increase in the potassium permeability of liver cell membranes (Haylett & Jenkinson, 1972a). This effect on membrane permeability appears to be mediated by α-adrenoceptors, as is that in intestinal smooth muscle (Jenkinson & Morton, 1967; Bülbring & Tomita, 1969), whereas glucose release from guinea-pig and rabbit liver slices is increased by activation of either α- or β-adrenoceptors (Haylett & Jenkinson, 1972b; Haylett, 1976).

Attempts to classify the receptors more exactly revealed a curious phenomenon. If α-receptors alone were to control the effect on potassium permeability, isoprenaline should increase potassium loss and membrane potential only at high concentrations, if at all. While this was usually the case, a few preparations were unexpectedly responsive to isoprenaline. Further, a chance observation showed that even unresponsive preparations became sensitive to isoprenaline if it was applied up to 20 min after the tissue had been exposed to the selective α-receptor agonist, amidephrine. The aim of the present work was to examine the pharmacological characteristics of this phenomenon in the hope of finding out more about the mechanism of action of catecholamine receptors. Preliminary accounts of some of the results have already been published (Haylett & Jenkinson, 1973; Koller, 1976).

## Methods

All experiments were carried out at 38°C with liver slices prepared from male guinea-pigs of the Porton or Hartley strains. Since the liver is enclosed in a capsule of connective tissue, the first slice from the surface was discarded. Care was also taken to avoid the region containing large blood vessels in the centre of the lobe.

Up to 8 slices (250–350 μm thick) were prepared for each experiment and transferred immediately to a 50 ml conical flask containing 25 ml of the basic incubation fluid. This had the composition (mM): NaCl 125, KCl 6, $CaCl_2$ 1, $MgSO_4$ 1.2, $NaH_2PO_4$ 1, $NaHCO_3$ 15, Na pyruvate 2, and was bubbled with a 95% $O_2$ and 5% $CO_2$ gas mixture. The flask and its contents were then placed in a bath at 38°C and shaken at 120–140 strokes/minute. After 30 min the slices were transferred to fresh incubation fluid, and again after a further 60 minutes. After a total incubation of 3 h a single slice was taken and pinned with stainless-steel needles to a convex Perspex platform. This was mounted in a water-jacketed Perspex bath (total volume 1 ml) through which a continuous stream of pre-warmed incubation fluid was fed by gravity from a reservoir held some 60–70 cm above the bath. Other reservoirs contained the various drug solutions to be tested and could be connected to the bath by a two-way tap. The flow-rate was maintained at 5–10 ml/min throughout (but for the brief interruption when the tap was turned) so that changes in the composition of the fluid bathing the slice were largely complete within 45 seconds.

In some experiments, as indicated in the text, the normal fluid was replaced by a chloride-free variant containing (mM): Na isethionate 125, K methylsulphate 6, $CaSO_4$ 1.5, $MgSO_4$ 1.2, $NaH_2PO_4$ 1, $NaHCO_3$ 15, and sodium pyruvate 2. This was also gassed with 5% $CO_2$ in $O_2$.

Further details of the preparation and incubation of the slices, and of their ionic compositions in both normal and chloride-free fluids, can be found in an earlier paper (Haylett & Jenkinson, 1972a).

### *Electrical recording*

Membrane potentials were measured in the conventional way with glass microelectrodes filled with potassium chloride (3 M, acidified to pH 2.5–4). Electrode resistances ranged from 10 to 35 MΩ. The potentials were displayed on an oscilloscope (Tektronix 502A) and on a heated-stylus chart recorder (Devices M2). The tracings shown are from the latter. As in earlier work (Haylett & Jenkinson, 1972a), the recordings were generally made from the second or third cell encountered on lowering the microelectrode into the slice. It was often possible to 'hold' the same cell for over 30 min and on occasion for as long as 90 minutes. All the results to be described are based on comparisons of the effects of drugs on continuously-recorded membrane potentials. This was thought preferable to a technique based on sampling from different cells, partly because the time course of the responses to drugs could be observed more easily, and also in view of the known heterogeneity of liver cells. It is likely that most, if not all, of the present recordings were from parenchymal cells since these make up from 85 to 95% of the cell mass of the liver (see discussion by Haylett & Jenkinson, 1972a). This is also consistent with the demonstration (Green, Dale & Haylett, 1972) that isolated parenchymal cells maintained under short-term tissue culture conditions have the same resting potential (−30 to −40 mV) as that observed in the present work, and show similar changes in membrane potential in response to the application of sympathomimetic amines.

### *Materials and drugs*

Inorganic salts of Analar quality were used in the preparation of bathing solutions, and were made up in glass-distilled water which had been passed through a column of mixed-bed ion exchange resin (Zerolit DM-F). Sodium isethionate was obtained from Koch-Light, potassium methylsulphate from Hopkins & Williams, and sodium pyruvate from Sigma.

Drugs used were (−)-noradrenaline bitartrate and (−)-phenylephrine hydrochloride (Koch-Light); adenosine; adenosine 5′-monophosphoric acid (AMP, sodium salt); adenosine 3′,5′-cyclic-monophosphoric acid (cyclic AMP, sodium salt); adenosine 5′-diphosphate (ADP, sodium salt); adenosine 5′-triphosphate (ATP, sodium salt); naphazoline hydrochloride; (±)-propranolol hydrochloride and papaverine (Sigma); theophylline hydrate (BDH); phentolamine mesylate and xylometazoline hydrochloride (Ciba).

The following were gifts, (±)-amidephrine mesylate and (−)-amidephrine hydrochloride (Mead Johnson); (−)-isoprenaline bitartrate (Ward & Blenkinsop); oxymetazoline hydrochloride and salbutamol sulphate (Allen & Hanbury); methoxamine hydrochloride (Burroughs Wellcome); tetrahydrozoline hydrochloride (Pfizer); tramazoline hydrochloride (Boehringer Ingelheim); thymoxamine hydrochloride (William R. Warner) and ICI 63-197 (2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazololo (1,5-a) pyrimidine).

Stock solutions (1–10 mM) of drugs were made up in distilled water just before each experiment began. Final dilutions were made a few minutes before use. Cyclic AMP, adenosine, AMP, ADP and ATP were dissolved in the appropriate volume of bathing fluid, again just before use.

## **Results**

In contrast to α-adrenoceptor agonists, isoprenaline generally causes only a small and inconsistent increase in the membrane potential of cells in guinea-pig liver slices (Haylett & Jenkinson, 1972b). However, it was found that following a 1–2 min application of the α-agonist amidephrine, low concentrations (10–50 nM) of isoprenaline produced substantial hyperpolarizations. This increase in responsiveness (which

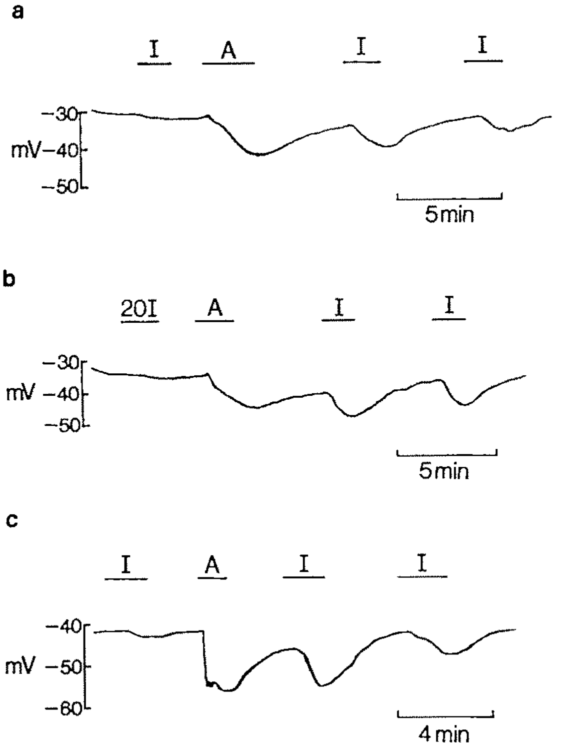

**Figure 1** (a) Potentiation by the α-adrenoceptor agonist (−)-amidephrine (10 μM, at A) of the effect of (−)-isoprenaline (50 nM, at I) on the membrane potential recorded from a cell in a guinea-pig liver slice. (b) As in (a) except that the first application of isoprenaline was at a 20-fold higher concentration (1 μM, at 20I). (c) As in (a) but from another preparation bathed in chloride-free (isethionate) solution.

for brevity will be referred to as 'potentiation') is illustrated in Figure 1, and was seen in all but 3 of 83 preparations tested, each from a different guinea-pig. It became particularly striking when the initial response to isoprenaline was negligible. An example of this is shown in Figure 1b; after amidephrine, isoprenaline at 50 nM produced a much greater hyperpolarization than did a 20-fold larger concentration beforehand.

A full analysis of potentiation would require the construction of dose-response curves for isoprenaline applied before, together with, and at various times after a range of concentrations of amidephrine. This has not been attempted in the present work. Our aim was instead to establish the conditions under which potentiation could be seen, and to examine some of its pharmacological characteristics, with a view to understanding the underlying mechanism.

As shown in Figure 1c, the phenomenon still occurred when the chloride content of the bathing fluid was replaced by the larger and presumably less permeant anion isethionate. Since the hyperpolarizations caused by sympathomimetic amines become larger and more rapid in both onset and decline in the absence of chloride (Haylett & Jenkinson, 1972a, b), it was often more convenient to study potentiation under these conditions. As in the earlier work, it was noted that fewer cells failed to respond to isoprenaline before amidephrine when the slices had been equilibrated in chloride-free solution. Nevertheless, potentiation was still easy to observe. Thus before amidephrine (20 μM of the (±)-isomer, or 10 μM of the (−)-isomer), (−)-isoprenaline (50 nM) produced a hyperpolarization of 4.9 ± 0.4 mV (s.e. mean, $n = 23$). This rose to 12.8 ± 1.0 mV ($n = 23$) 3–5 min after amidephrine.

The aim of the main series of experiments was to test whether potentiation could also be observed with other α- and β-agonists. Cyclic AMP was examined as well, in view of its suggested role in many β-mediated responses. The results have been summarized in Table 1, and will be discussed under the listed headings.

**Table 1** Agents that either potentiated the effects of other agonists on the membrane potential of cells in guinea-pig liver slices or whose effects could be potentiated

| A | | | B |
|---|---|---|---|
| *Agents able to cause potentiation* | | | *Agents whose effects could be potentiated* |
| (i) | (ii) | (iii) | |
| Amidephrine | Noradrenaline | ADP | Isoprenaline |
| Naphazoline | Phenylephrine | ATP | Salbutamol |
| Oxymetazoline | Methoxamine | | Cyclic AMP |
| Tramazoline | | | |
| Tetrahydrozoline | | | |
| Xylometazoline | | | |

See text for explanation of the sub-categories (i), (ii) and (iii).

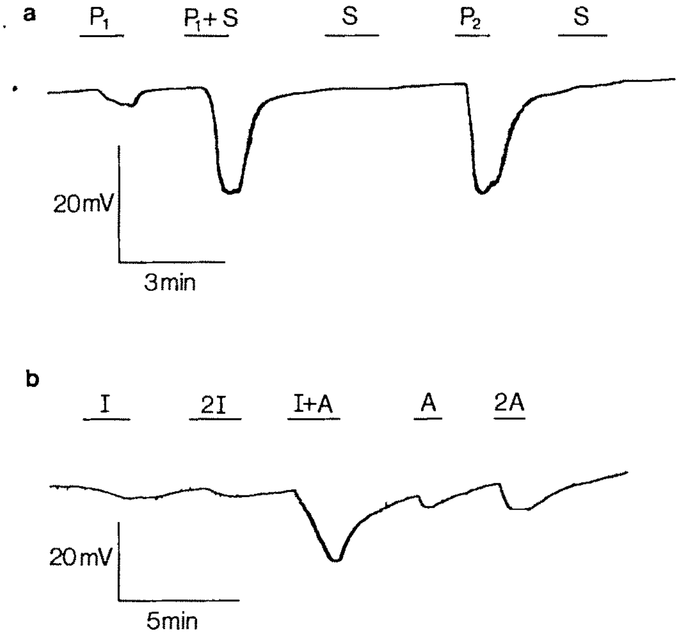

**Figure 2** (a) Membrane potential responses to low doses of (−)-phenylephrine (2 µM, at $P_1$) and salbutamol (0.2 µM, at S) applied separately and in combination (at $P_1$+S); also shown is the inability of a higher concentration of (−)-phenylephrine (8 µM, at $P_2$) to potentiate the response to a subsequent application of salbutamol; chloride-free conditions. (b) Responses from another preparation to low doses of (−)-isoprenaline (25 nM, at I) and (−)-amidephrine (2 µM, at A) applied separately and in combination (at I+A). Also illustrated are the responses to twice the concentration of both (−)-isoprenaline (50 nM, at 2I) and (−)-amidephrine (4 µM, at 2A).

*Agents that caused potentiation*

The sympathomimetic amines listed in columns (i) and (ii) of Table 1 were chosen for their known $\alpha$-agonist properties (Mujić & van Rossum, 1965; Sanders, Miller & Patil, 1975). All were able to cause both potentiation and hyperpolarization in the dose range 1–50 µM. However, an interesting difference was seen between amidephrine and the various imidazolines on the one hand, and noradrenaline, phenylephrine and methoxamine (column ii) on the other. That is, an increased response to a $\beta$-agonist (or to cyclic AMP, see later), could be detected at any time up to 15 to 30 min after a single application of one of the first group of $\alpha$-agonists, whereas members of the second group caused potentiation only when applied together with the $\beta$-agonist, as illustrated for phenylephrine in Figure 2a.

Figure 2b shows a control experiment to demonstrate that the increased response seen on applying an $\alpha$- and a $\beta$-agonist together is not simply a consequence of a non-linear relationship between the concentration of a hyperpolarizing agent and the resulting rise in membrane potential. Thus doubling the concentration of either amidephrine or isoprenaline caused a smaller increase in response than did combining the low doses of each agonist.

The finding that all the $\alpha$-agonists tested caused both hyperpolarization and potentiation suggested these actions were $\alpha$-adrenoceptor mediated. In keeping with this, both effects could be reversibly inhibited by phentolamine at 10 µM, as shown in Figure 3. That this relatively high concentration is required agrees with previous results (Haylett & Jenkinson, 1972b) which had shown that phentolamine is much less effective in blocking responses to $\alpha$-agonists in guinea-pig liver than in other tissues. Thus the dose-ratio with phentolamine at 10 µM was only 13 in the earlier study, as compared with the value of more than 500 to be expected from measurements with a variety of smooth muscles (see e.g. Sheys & Green, 1972). A similar discrepancy with the $\alpha$-antagonist thymoxamine was seen in the present work. This agent, when tested at 10 µM against the hyperpolarizing action of amidephrine, gave a dose-ratio of 11 in one experiment, and 13 in another, whereas a value of at least 100 would have been expected from studies with smooth muscle from several species, including the guinea-pig (Birmingham & Szolcsányi, 1965). In view of these differences

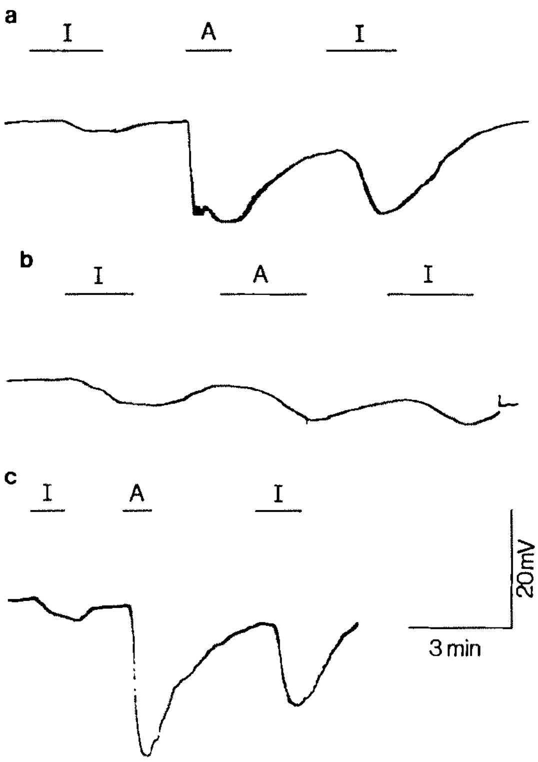

**Figure 3** The effect of phentolamine on the potentiation of the response to (−)-isoprenaline (50 nM, at I) by (−)amidephrine (10 μM, at A); chloride-free conditions. (a) Control responses; (b) in the presence of phentolamine (10 μM) applied 3 min before the first dose of isoprenaline; (c) 70 min after washout of the antagonist.

between liver and other tissues it would seem best to continue to describe the receptor concerned in the liver as 'α-like' until its pharmacological characteristics have been studied further.

### *Agents that could be potentiated; salbutamol and cyclic AMP*

Agents that could be potentiated by prior or simultaneous application of α-agonists are listed in Table 1B. As Figure 4 illustrates, the phenomenon was as clearcut with salbutamol as with isoprenaline (see also Figure 10). Since salbutamol is not a substrate for catechol-*O*-methyltransferase, this finding rules out the rather unlikely possibility that the increased response to isoprenaline was a consequence of a transient inhibition of this enzyme by the α-agonist, leading in turn to an increase in isoprenaline concentration in the vicinity of the β-receptors. It seemed more likely that potentiation reflected a change either in the process of β-receptor activation, or in one or more of the steps which follow activation. One of the earliest of these steps in guinea-pig liver, as in many other tissues, is an increase in the intracellular cyclic AMP concentration (see Osborn, 1975). It was interesting for this reason to test whether externally applied cyclic AMP would also hyperpolarize guinea-pig liver cells, as has been demonstrated in rat liver (Friedmann, Somlyo & Somlyo, 1971; Dambach & Friedmann, 1974) and, if so, to determine whether the response became greater after an α-agonist had been applied.

It was found that under the usual experimental conditions, cyclic AMP caused only a small increase in membrane potential; at 1 mM the hyperpolarization was barely detectable (1.2 ± 0.3 mV, $n=9$). As Figure 5a shows, the response became much larger after amidephrine, just as had been observed with β-agonists. Similar results (Figure 5b) were obtained in chloride-free solution though it was noted that cyclic AMP, like isoprenaline, became more effective under these conditions. Thus, before amidephrine, cyclic AMP (0.5 mM) produced a hyperpolarization of 4.5 ± 0.5 mV ($n=10$). However, potentiation was still striking; after amidephrine (10 μM of the (−)-isomer) the same concentration

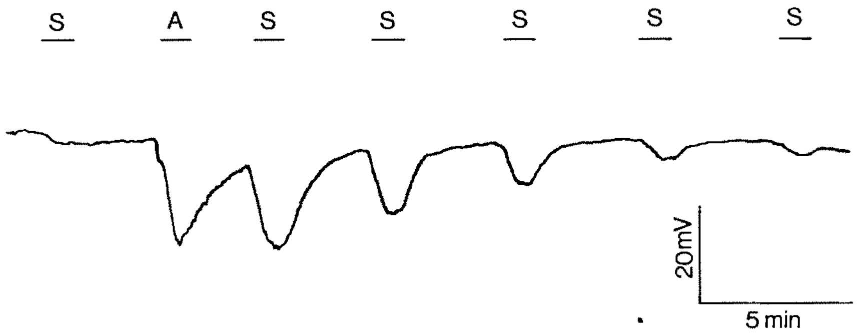

**Figure 4** Potentiation by (±)-amidephrine (20 μM, at A) of the effect of salbutamol (0.5 μM, at S) on the membrane potential: chloride-free conditions.

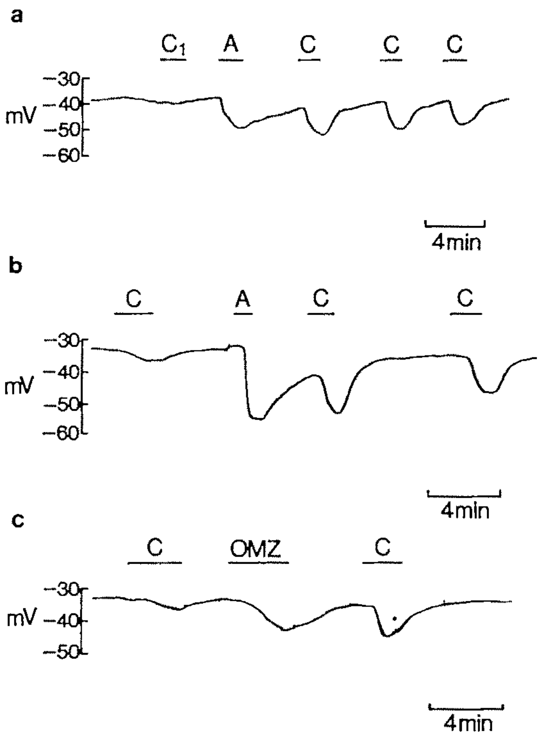

Figure 5 (a) Potentiation of the membrane potential responses to cyclic AMP (2 mM, at $C_1$: 0.5 mM at C) by (–)-amidephrine (10 µM, at A). (b) From another preparation bathed in chloride-free medium; although responses to cyclic AMP (0.5 mM, at C) are larger under these conditions, potentiation following (–)-amidephrine (10 µM, at A) is still clear-cut. (c) As in (b) but showing potentiation of cyclic AMP by the $\alpha$-agonist oxymetazoline (2 µM, at OMZ).

of cyclic AMP caused a hyperpolarization of $13.1 \pm 0.9$ mV ($n = 9$). This could as readily be shown with other $\alpha$-agonists, for example oxymetazoline (see Figure 5c). The response to cyclic AMP before and after amidephrine was unaffected by (±)-propranolol at 1 µM, a concentration sufficient to reduce greatly the effects of isoprenaline (50 nM) on glucose release from guinea-pig liver slices, and on $^{42}$K efflux both before and after this $\alpha$-agonist (Haylett & Jenkinson, 1972b; 1973).

It is commonly assumed that responses to externally applied cyclic AMP reflect an increase in the concentration of the nucleotide within the cell. This assumption is perhaps better founded for the liver than for other tissues, since liver cell membranes are appreciably permeable to cyclic AMP (Exton, Lewis, Ho & Park, 1972; Strange & Percy-Robb, 1975). Accepting that externally applied cyclic AMP does increase the concentration in the cytoplasm, the finding that it produces a greater hyperpolarization after amidephrine is clearly in keeping with the idea that potentiation of the response to a $\beta$-agonist occurs at a stage subsequent to both $\beta$-adrenoceptor and adenylate cyclase activation.

*Effects of other adenine nucleotides*

Quite different results were obtained with other adenine nucleotides. ADP and ATP markedly hyperpolarized guinea-pig liver cells, whereas AMP and adenosine were inactive at concentrations up to 1 mM, even after amidephrine. Figure 6a summarizes the effects of these compounds on membrane potential, together with those of cyclic AMP and (–)-amidephrine. ADP and ATP appeared to be about 250 and 160 times less active respectively than (–)-amidephrine, although these potency differences could well be over-estimates. This is because the responses to ADP and ATP were somewhat different in time course from those to amidephrine, being faster in both onset and decline and often 'fading' during the test exposure. These differences were particularly clear-cut in chloride-free solution, as illustrated in Figure 6b. The maximum responses to ADP and ATP may therefore have been underestimated since 'fade' may have been substantial by the time that the agonist had equilibrated with the tissue (see e.g. Paton & Waud, 1964). From Figure 7 it can be seen that amidephrine caused no change in the responses to ADP or ATP at a time when $\beta$-mediated responses were substantially potentiated. With this result in mind, it was interesting to test whether these agents could themselves increase the response to a $\beta$-agonist. Figure 8 shows that ADP and ATP resembled noradrenaline, methoxamine and phenylephrine in that each caused potentiation, but only when applied together with the $\beta$-agonist. However, at doses producing the same hyperpolarization, both nucleotides seemed less effective in this regard than $\alpha$-agonists. This could reflect the more transient nature of the ADP and ATP responses.

*The effects of phosphodiesterase inhibitors*

Cyclic AMP is inactivated by the enzyme phosphodiesterase, of which at least two forms exist in the liver, one membrane bound and the other in the cytoplasm (Russell, Terasaki & Appleman, 1973; Appleman & Terasaki, 1975). Thus agents such as papaverine, theophylline and ICI 63-197 (see Davies, 1973) which inhibit phosphodiesterase would in general be expected to increase responses mediated by cyclic AMP in the liver, as elsewhere. In keeping with this, the hyperpolarization caused by salbutamol (100 nM) became greater and more rapid in onset in the presence of papaverine (25 µM). This was seen in four of the five preparations tested; although the dose-response relation for papaverine was not explored in

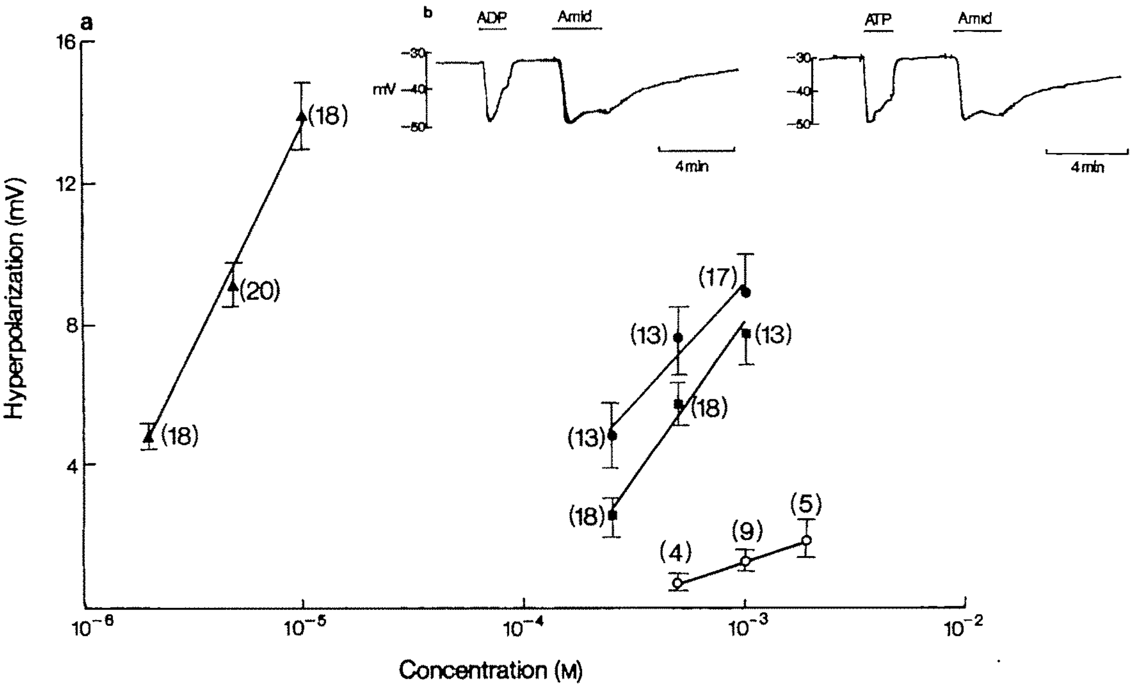

Figure 6 (a) Dose-response relations for the effects on membrane potential of (−)-amidephrine (▲), ATP (●), ADP (■) and cyclic AMP (○). The values plotted are the means, ± s.e. of the number of observations shown in brackets. Normal bathing solution. (b) Comparisons of the hyperpolarizations caused by (−)-amidephrine (5 μM, at Amid), ADP (0.4 mM) and ATP (0.4 mM) in chloride-free medium where 'fading' of the responses to ADP and ATP was particularly evident (see text). Records from the same cell.

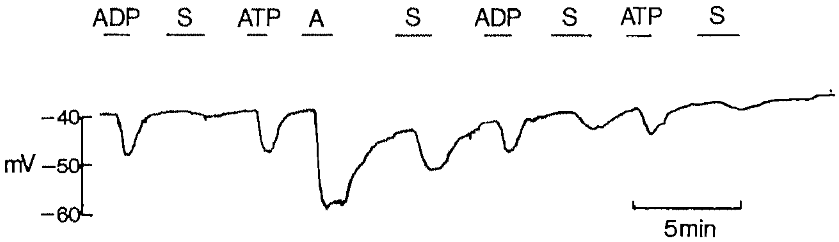

Figure 7 The effects of (−)-amidephrine (10 μM, at A) on responses to salbutamol (0.2 μM, at S) and to low doses of the adenine nucleotides ADP (0.25 mM) and ATP (0.125 mM). The α-agonist potentiates only the responses to the β-agonist. Chloride-free conditions.

any detail, the effect was noticeable at 5 μM and became maximal at about 25 μM. However, as illustrated in Figure 9a, this potentiation was not maintained, and indeed was followed by a period of inhibition even when papaverine had been withdrawn. In two of the four responsive preparations, the recovery was incomplete even after the slice had been washed with papaverine-free solution for more than an hour.

In the presence of papaverine, again at 25 μM, the hyperpolarization caused by low doses of the α-agonist amidephrine (0.5 μM) also sometimes became larger and more rapid in onset (see Figure 9b). Of eight preparations tested in this way, four showed reversible increases in response, two showed increased responses followed by inhibition, and the remainder were unaffected. In a few preliminary experiments theophylline (100–400 μM) and ICI 63-197 (25–50 μM) gave qualitatively similar results to papaverine. That phosphodiesterase inhibition should alter the response to an α-agonist such as amidephrine might seem surprising since, in contrast to

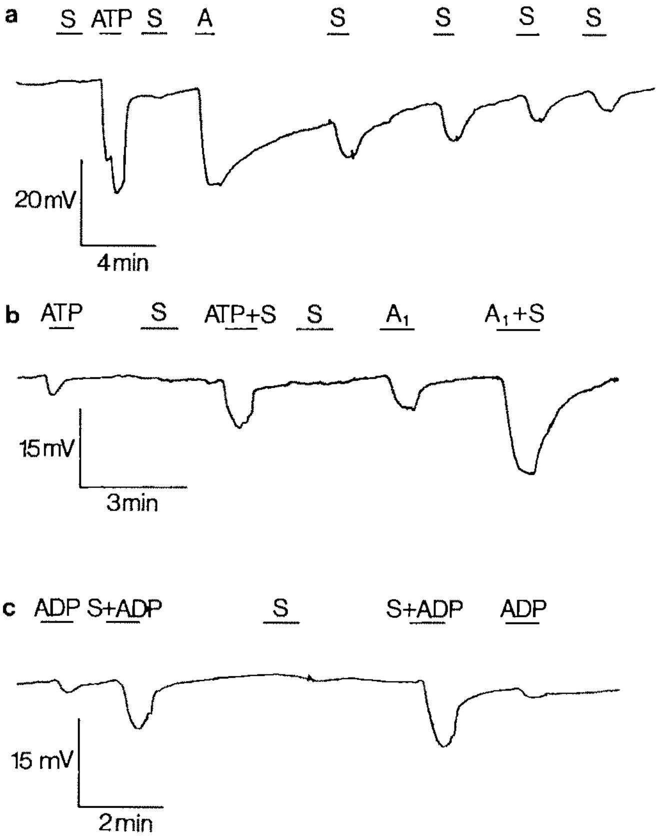

**Figure 8** (a) The effects of ATP (0.5 mM) and (−)-amidephrine (10 μM, at A) on the responses to salbutamol (0.1 μM, at S). Whereas (−)-amidephrine is able to potentiate subsequent salbutamol responses, ATP is ineffective in this respect; chloride-free conditions. (b) Responses to low doses of ATP (50 μM) and salbutamol (0.2 μM, at S) applied separately and in combination (at ATP+S). Also shown for comparison are responses to (−)-amidephrine applied alone (4 μM, at $A_1$) and together with this dose of salbutamol (at $A_1$ + S); chloride-free conditions. (c) The effects of low doses of ADP (0.1 mM) and salbutamol (0.2 μM, at S) applied separately and in combination (at ADP+S). From the same preparation as (b) and under the same conditions.

isoprenaline, amidephrine does not increase cyclic AMP levels in guinea-pig liver slices (Osborn, 1975). A possible explanation is that adenylate cyclase has a basal activity even in the absence of agonists. If this is so, inhibition of phosphodiesterase should cause cyclic AMP to accumulate, as though a $\beta$-agonist had been applied. The increased response to amidephrine (cf. Figure 9) would then be equivalent to that observed when amidephrine is applied together with a $\beta$-agonist.

However, the finding that the potentiating action of phosphodiesterase inhibitors often changed to inhibition made it clear that additional factors must have been at work. Whatever the explanation for this effect, its occurrence evidently limits the value of phosphodiesterase inhibitors (certainly those so far examined) as tools for the study of the membrane actions of catecholamines in this tissue.

### *The role of electrogenic transport*

A conceivable explanation for potentiation was that it might be associated in some way with changes in electrogenic transport. This did not seem very probable since earlier work had shown that although electrogenic transport can occur in guinea-pig liver slices, it does not underlie the hyperpolarizations caused by noradrenaline and isoprenaline. The main evidence for the existence of electrogenic transport is that restoration of external potassium to liver slices bathed in a nominally potassium-free solution increases the membrane potential, an effect which is reversibly abolished by ouabain. In contrast, the hyperpolarizations caused by noradrenaline and isoprenaline are insensitive to ouabain at the same concentration, and are better accounted for in terms of an increase in the

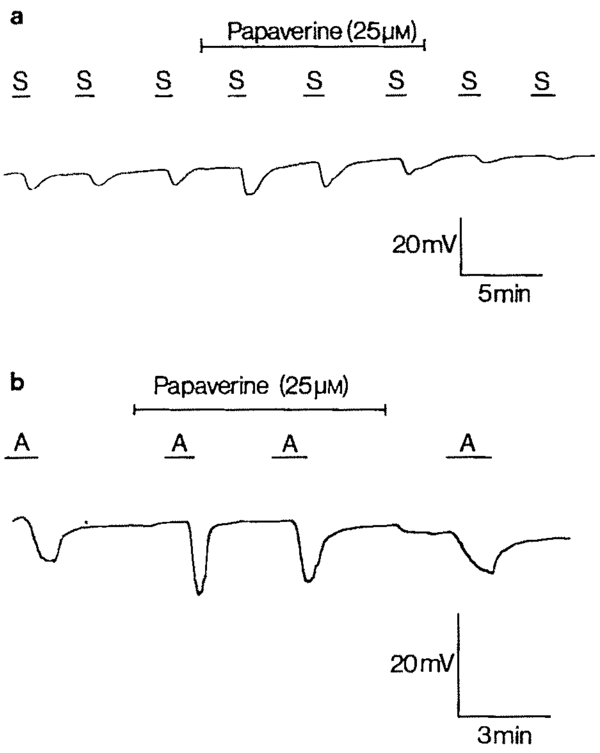

**Figure 9** The effects of papaverine (25 µM) on membrane potential responses to (a) salbutamol (0.1 µM, at S) and (b) (±)-amidephrine (5 µM, at A); chloride-free conditions.

potassium permeability of the cell membrane (Haylett & Jenkinson, 1972b; 1973).

Nevertheless it seemed worth testing whether electrogenic transport became more pronounced at a time when the response to a β-agonist had been increased by prior application of an α-agonist. The outcome of such an experiment is illustrated in Figure 10, where it can be seen that the hyperpolarization resulting from potassium restoration was little changed at a time when potentiation was clearly evident.

Thus potentiation does not seem to involve a change in potassium-dependent electrogenic transport. Similarly the fact that potentiation continues in slices bathed in chloride-free solutions rules out mechanisms based on changes in chloride transport.

## Discussion

The first of the two main findings is that 'potentiation' in guinea-pig liver is not a specific feature of the agonist pair amidephrine/isoprenaline with which it was initially observed. It has now been shown to occur with a range of sympathomimetic amines, including some which are not substrates for various processes for the uptake and inactivation of catecholamines. This makes it unlikely that changes in drug concentration in the immediate vicinity of the receptors could account for the increase in the response to a β-adrenoceptor agonist applied shortly after an α-agonist. The second main finding, i.e. that the hyperpolarization caused by externally applied cyclic AMP also became larger after an α-agonist, is in keeping with this.

A more puzzling observation was that some agents, e.g. noradrenaline and ATP (see Table 1, columns ii and iii) potentiated the response to isoprenaline and salbutamol only when applied at the same time whereas even a brief exposure to amidephrine or the various imidazolines (column i of Table 1) was followed by an increase in β-responsiveness which lasted for many minutes. This may have been simply a consequence of the rather longer duration of action of the latter α-agonists, as judged by the rate at which the hyperpolarization they elicited subsided after washout. However, potentiation could still be detected even when the membrane potential had returned to its previous level. The possibility remains therefore that

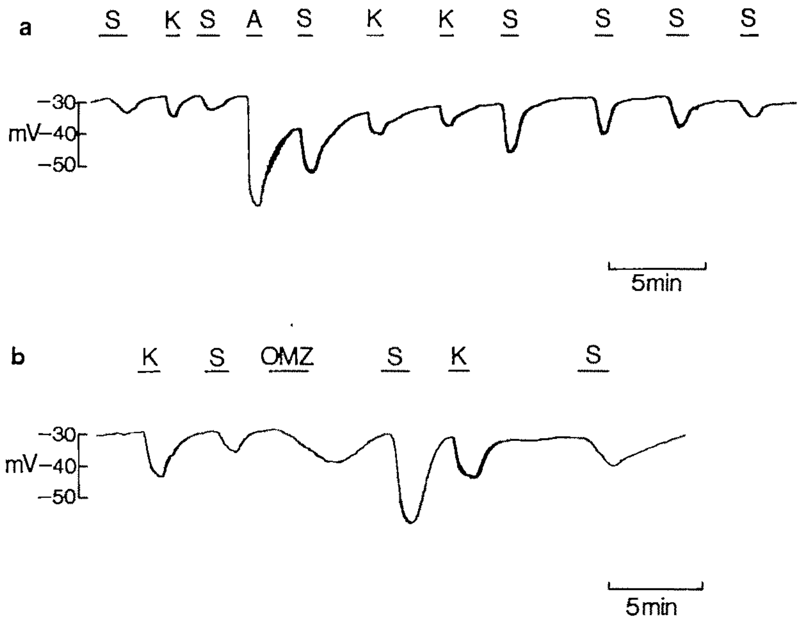

**Figure 10** (a) Continuous record of the membrane potential of a cell in a liver slice bathed in a potassium and chloride-free solution (chloride replaced by isethionate, potassium by sodium). Brief re-introduction of potassium (6 mM, at K) caused hyperpolarization (see text). In contrast to the hyperpolarization caused by salbutamol (0.5 μM, at S), this response to potassium was no greater after (±)-amidephrine (20 μM, at A). (b) As in (a) but with oxymetazoline (1 μM, at OMZ) instead of (±)-amidephrine. From the same preparation, but 25 min later.

those $\alpha$-agonists which cause prolonged potentiation act in a qualitatively different way from the other agents, and this requires further study.

It was also found that the adenine nucleotides ADP and ATP cause a transient dose-dependent increase in the membrane potential of guinea-pig liver cells. Although the mechanism of this hyperpolarization has yet to be examined, the fact that the response to these nucleotides was not increased by $\alpha$-agonists suggests, albeit indirectly, that changes in cyclic AMP are not involved. Indeed, the additional finding that ADP and ATP can potentiate the response to a $\beta$-agonist suggests that the action of both nucleotides may have more in common with $\alpha$- than with $\beta$-receptor activation. ATP-induced hyperpolarizations have also been described in smooth muscle by Tomita & Watanabe (1973), and these authors favour an explanation based on an increase in the potassium permeability of the membrane; the effects of ADP and ATP on membrane potential in the liver may possibly arise in the same way. Whatever the mechanism, the finding that compounds other than $\alpha$-agonists are capable of potentiating the response to a $\beta$-agonist strongly suggests that the observed interactions involve not the $\alpha$- and $\beta$-receptors *per se*, but one or more of the steps which follow their activation.

Since several of these steps are as yet only partly understood, further discussion is necessarily speculative. However, there are two pieces of evidence which suggest possible lines for such speculation to follow. Firstly, $\beta$-agonists increase the cyclic AMP content of guinea-pig liver slices (Osborn, 1975), and since the response to applied cyclic AMP itself is facilitated by $\alpha$-agonists, it seems possible that potentiation reflects a change in the way in which the cell responds to an increase in the concentration of this nucleotide in the cytoplasm. Secondly, recent work by Haylett (1976) has shown that $\alpha$-agonists (amidephrine and noradrenaline) cause a large though transient rise in the efflux of labelled calcium from guinea-pig and rabbit liver slices, isoprenaline being much less effective under normal conditions. Though the interpretation is still tentative, this $\alpha$-mediated increase in calcium efflux may reflect a rise in the concentration of calcium ions in the cytoplasm. This increase in calcium ion concentration could in turn underlie the observed rise in potassium permeability, as has been described for other tissues, including erythrocytes, salivary glands, and several vertebrate and invertebrate neurones (see Berridge, 1975, for references).

Since the 'potentiated' membrane response to $\beta$-

activation is so similar to the α-action (in each instance, there is an increase of similar time course in membrane potential and potassium efflux—see Haylett & Jenkinson, 1973), it is tempting to suppose that when potentiation occurs, cyclic AMP is now able to increase cytoplasmic calcium, though by a different mechanism from that which operates during α-activation. In the absence of any direct evidence to indicate the source of this calcium it is clearly premature to advance detailed suggestions about the two postulated mechanisms for calcium release, or about their interactions. Nevertheless, it should be mentioned that in other tissues, conditions exist in which two 'cellular' calcium pools interact to give rise to an enhanced, even regenerative, calcium release (e.g. Fabiato & Fabiato, 1975). Also, both isoprenaline and externally applied cyclic AMP cause a much greater increase in $^{45}$Ca efflux from guinea-pig liver slices when applied after an α-agonist (D.G. Haylett, D.H. Jenkinson & K. Koller, unpublished observations), which is in keeping with the above suggestions (see also Kroeger & Marshall, 1973).

Another possible explanation for potentiation could be envisaged if cyclic AMP were to exert a more direct effect on the ionic permeability of the liver cell, as has been suggested for other inexcitable tissues e.g. avian erythrocytes (see Gardner, Mensh, Kiino & Aurbach, 1975 for references). The relative inability of cyclic AMP (whether externally applied, or resulting from β-receptor activation) to affect membrane potential in untreated slices might then be explained if, as it seems reasonable to suppose, there is a basal level of phosphodiesterase activity which serves to keep down the concentration of cyclic AMP within the cells, and more particularly, at the inner surface of the membrane.

If activation of the α-receptors in some way depresses this basal phosphodiesterase activity, subsequent application of a β-agonist (or of cyclic AMP) could result in an increase in cyclic AMP at the membrane, and hence in a rise in potassium permeability, and membrane potential. Since potentiation of β-mediated responses can be seen with both α-agonists and the adenine nucleotides ADP and ATP it is difficult to imagine that all these agents could directly inhibit phosphodiesterase, but an indirect effect (e.g. through increased levels of intracellular calcium) might be envisaged. In principle, this should have been testable by the application of selective phosphodiesterase inhibitors which on such a scheme would be expected to increase greatly the responses to cyclic AMP and to β-agonists, and at the same time to reduce or abolish potentiation, as defined in the present work. Unfortunately, the results obtained with phosphodiesterase inhibitors were complex; though the predicted rise in the response to β-agonists was observed, it was quickly followed by a long-lasting inhibition, of unknown origin. The experiments did not therefore allow us to distinguish between these possible explanations for potentiation, which are indeed only two of several that can be proposed on the basis of the diverse interactions that occur between intracellular calcium and the enzymes concerned in the formation, action and destruction of cyclic nucleotides (for reviews, see Rasmussen & Goodman, 1975; Berridge, 1975). More will have to be known about these interactions, and about the direct and indirect effects of cyclic nucleotides on liver cell membranes, before the point can be settled.

### *Possible implications*

Although we have not tested whether potentiation occurs in other *in vitro* situations or *in vivo*, there are interesting parallels between the present findings and some recent reports on the effects of sympathomimetic amines on plasma potassium in dogs. It has long been known that adrenaline causes a biphasic change in plasma potassium in several species, an early rise being followed by a prolonged fall. While it is clear that the initial rise is mainly due to a net loss of potassium from the liver (D'Silva, 1936; 1937; Todd & Vick, 1971; Vick, Todd & Luedke, 1972), there is conflicting evidence on the type of receptors concerned in this action. The finding (e.g. Todd & Vick, 1971) that the hyperkalaemia in the dog can be abolished by α-blocking agents suggest that it is α-mediated, as in the cat (Ellis & Beckett, 1963). However, this has not been easy to reconcile with the parallel observation, also in the dog, that the initial hyperkalaemia is either partially (Grassi, de Lew, Cingolani & Blesa, 1971) or almost completely (Todd & Vick, 1971) inhibited by propranolol (though see also Castro-Tavares, 1976). A further puzzle is that in this species combined administration of low doses of an α- and a β-agonist (phenylephrine and isoprenaline respectively) causes a 'super-additive hyperkalaemia' i.e. an increase in plasma potassium much greater than the sum of the separate responses to each drug (Todd & Vick, 1971; Castro-Tavares, 1975). These authors have favoured explanations based on β-mediated changes in blood flow; the increased loss of potassium is considered to result either from more effective 'washout' from the liver (Todd & Vick, 1971), or from increased hepatic hypoxaemia due to β-mediated opening of vascular shunts within the liver (Castro-Tavares, 1975). However, the present finding that simultaneous activation of α- and β-receptors caused a more than additive increase in membrane potential in an isolated preparation (e.g. Figures 2a,b and 8b) makes it tempting to seek at least a partial explanation of the *in vivo* findings in terms of an interaction at the level of the liver cells themselves. Should potentiation be found to occur in intact liver, or indeed in other situations, it is clear that receptor classification studies would require particular care. For example, an α-adrenoceptor antagonist might be found to reduce the response to a β- as well as to an α-agonist (see Figure 3). The finding that α-agonists can sometimes potentiate a β-response also raises the more general point that the effects of adrenaline and noradrenaline (which activate both receptors) may not always be equivalent to the sum of the responses to selective activation of each receptor.

We are grateful to Professor J.W. Black, Dr D.G. Haylett and Dr R. Niedergerke for helpful discussions, and to Mrs Margaret Butler for skilful technical assistance. K.K. held an M.R.C. studentship during the course of the work. We would like to thank Mead Johnson, Ward & Blenkinsop, Allen & Hanbury, Burroughs Wellcome, Pfizer, Boehringer Ingelheim, William R. Warner and ICI for gifts of drugs mentioned in the Methods section.

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*(Received June 23, 1976*
*Revised August 13, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 177–189

# THYROID HORMONE-DEPENDENT INTERCONVERSION OF MYOCARDIAL $\alpha$- AND $\beta$-ADRENOCEPTORS IN THE RAT

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1 The effects of thyroid state on the properties of adrenoceptors mediating inotropic and chronotropic responses of the rat heart were assessed on the basis of the relative potencies of $\alpha$- and $\beta$-adrenoceptor agonists, the effects of $\alpha$- and $\beta$-adrenoceptor antagonists and the tissue uptake of [$^3$H]-phenoxybenzamine ([$^3$H]-PB).

2 In isolated, electrically driven left atria the ratio of the inotropic potencies of isoprenaline and phenylephrine and the inhibitory potency of propranolol (40nM–4 μM) were significantly reduced after thyroidectomy and were moderately increased after thyroxine treatment of control rats.

3 Block of inotropic responses to noradrenaline and to phenylephrine by PB (7.3nM–7.3 μM) and the tissue uptake of [$^3$H]-PB were significantly greater in preparations from thyroidectomized than in those from control or from thyroxine treated rats. $\alpha$-Adrenoceptor inhibition by phentolamine (0.26–2.6 μM) also increased after thyroidectomy, and phentolamine effectively protected $\alpha$-adrenoceptors from block by and binding of [$^3$H]-PB.

4 The $\beta_1$-receptor antagonist H 93/26 (0.1 μM) significantly potentiated $\alpha$-adrenoceptor blockade by PB in hypothyroid but not in control preparations.

5 In spontaneously beating right atria the chronotropic potency of agonists and the effects of antagonists were altered in the same way as were inotropic responses and the slope of the agonist concentration-response curves were significantly reduced after thyroidectomy. Effects of agonists and antagonists were not significantly influenced by thyroxine treatment.

6 Changes in the effects and tissue uptake of sympathomimetic drugs observed after thyroidectomy were reversed to or beyond control levels by thyroid hormone treatment of thyroidectomized animals.

7 The results presented are interpreted as indicating a thyroid hormone-dependent interconversion of myocardial $\alpha$- and $\beta$-adrenoceptors. It is suggested that this interconversion is similar to that observed earlier in frog hearts at different temperatures, and that both effects may reflect an allosteric transition between two forms of a single basic structure.

## Introduction

The striking similarities between the effects of sympathetic activity and the cardiovascular symptoms of hyperthyroidism have long suggested some relationship. However, there has been considerable controversy in the literature about the influence of thyroid hormones on the adrenergic reactivity of the heart. Classical works suggested that hyperthyroidism increased and hypothyroidism decreased the sensitivity of the myocardium to catecholamines or to sympathetic nerve stimulation, although some of the more recent studies failed to confirm these changes (Harrison, 1964; Waldstein, 1966). Some of this controversy may have arisen from the fact that most studies did not distinguish between $\alpha$- and $\beta$-adrenoceptor responses and did not test the effects of drugs with selective actions on $\alpha$- and $\beta$-adrenoceptors. Although the adrenoceptors mediating inotropic and chronotropic responses of the heart have the pharmacological characteristics of $\beta$-adrenoceptors, there has been evidence that $\alpha$-adrenoceptors can also mediate these responses in both amphibian (Nickerson, 1949) and mammalian hearts (Wenzel & Su, 1966; Benfey & Varma, 1967; Govier, 1968), and the two kinds of receptors may be differently influenced by thyroid hormones. Recent studies with isolated hearts of frogs have shown that the adrenoceptors that mediate inotropic and chronotropic responses can be altered qualitatively by ambient temperature in a way that their characteristics shift from $\beta$ toward $\alpha$ when the temperature of the isolated hearts is reduced through a critical range of 17–22°C (Kunos & Szentiványi, 1968; Buckley & Jordan, 1970; Kunos, Yong & Nickerson, 1973; Harri, 1973; Kunos & Nickerson, 1976). These results suggested that $\alpha$- and $\beta$-adrenoceptors represent different forms of a single basic structure, whose properties can be influenced by the metabolic activity of the tissue. Although qualitatively similar changes in adrenoceptor characteristics appear to occur in the mammalian myocardium (Kunos & Szentiványi, 1968; Kunos, Vermes-Kunos, Boyd & Nickerson, 1973; Benfey, Kunos & Nickerson, 1974; Amer & Byrne, 1975), the physiological significance of a temperature-induced change in a homeothermic species is obviously limited. Several observations indicate, however, that adrenoceptors can be influenced by factors other than temperature, including the administration of a iodo- or fluoroacetate (Nickerson & Nomaguchi, 1950) or dinitrophenol (Kunos & Szentiványi, 1968; Matheny & Ahlquist, 1975), skeletal muscle activity (Szentiványi, Kunos & Juhász-Nagy, 1970a) and increased vagal influence on the myocardium (Szentiványi, Kunos & Juhász-Nagy, 1970b). Under these conditions, $\alpha$-adrenoceptor properties were always promoted by a decrease and $\beta$-adrenoceptor properties were enhanced by an increase in metabolic activity. Therefore, it seemed possible that changes in thyroid state would also alter adrenoceptor properties. The results presented in this paper indicate a maximal dominance of $\beta$-adrenoceptors mediating inotropic and chronotropic responses in rats after thyroid hormone treatment, and an increased dominance of $\alpha$-adrenoceptors after thyroidectomy. Some of these results have been published in a preliminary report (Kunos, Vermes-Kunos & Nickerson, 1974).

## Methods

Experiments were performed on isolated, electrically driven left and spontaneously beating right atria of male Sprague-Dawley rats. Hypothyroidism was induced by surgical thyroidectomy in rats weighing 180–220 grams. The thyroid glands were removed under ether anaesthesia. In most animals, the parathyroids could not be separated and were also removed. In a few rats, hypothyroidism was induced by a single intravenous injection of 200 μCi of $^{131}$I. The results obtained in preparations from these animals were identical with those in atria from surgically thyroidectomized rats and the two groups were pooled. The experiments on the isolated tissues were carried out 6 weeks after the operation or $^{131}$I treatment, by which time the animals had become hypothyroid. Hypothyroidism was ascertained by reduction of growth, dryness of fur, a significant decrease in serum thyroxine levels, as determined by the method of Murphy & Pattee (1964), and by a significant decrease in the control rate of beating of right atria (Table 1). Control preparations were from weight-matched, or from sham-operated age-matched animals. Responses of atria from the two kinds of controls were not significantly different and the results were pooled. A third group of animals was first thyroidectomized and then injected daily with 1 mg/kg thyroxine ($T_4$) or 0.25 mg/kg tri-iodothyronine ($T_3$) for 1 week before the experiment. The effects of $T_4$ or $T_3$ treatment on responses of the isolated atria were similar. In the last group of animals, high serum thyroxine levels were maintained (Table 1) by daily intraperitoneal injections of 1 mg/kg $T_4$ for 8 days before the experiment.

All animals were anaesthetized with ether and the

**Table 1** The effect of thyroid state on weight gain, serum thyroxine ($T_4$) levels, basal heart rate and on the ratio of the inotropic potency of isoprenaline and phenylephrine

| | *Weight gain*[a] *(g/week)* | *Serum $T_4$ levels*[b] *(ng/ml)* | *Basal right*[c] *atrial rate (beats/min)* | *Isoprenaline*[e] *phenylephrine molar potency ratio (log)* |
|---|---|---|---|---|
| Control | $28.2 \pm 1.4$ | $58.5 \pm 3.4$ | $192.1 \pm 4.2$ | $3.50 \pm 0.14$ |
| | (40) | (10) | (17) | (30) |
| Thyroidectomized | $3.2 \pm 0.6^{**}$ | $21.2 \pm 4.2^{**}$ | $118.2 \pm 3.1^{**}$ | $2.24 \pm 0.16^{**}$ |
| | (40) | (8) | (32) | (18) |
| Thyroidectomized + $T_3$ or $T_4$ | $11.9 \pm 5.7^{*}$ | $91.3 \pm 8.1^{**d}$ | $251.2 \pm 5.8^{**}$ | $4.02 \pm 0.24$ |
| | (7) | (6) | (15) | (6) |
| Control + $T_4$ | $20.5 \pm 6.8$ | $169.3 \pm 26.3^{**}$ | $253.2 \pm 8.2^{**}$ | $4.61 \pm 0.21^{**}$ |
| | (8) | (6) | (8) | (12) |

Values are means with their standard errors. Numbers of experiments are given in parentheses. Significance of difference from corresponding control values: $^{*}P < 0.05$, $^{**}P < 0.005$.

[a]during week prior to experiment; [b]determined at the time of the experiment by the method of Murphy & Pattee (1964); [c]at 31°C; [d]measured in animals treated with $T_4$; [e]for inotropic responses of left atria.

hearts quickly removed. The right and left atria were separated and mounted in 20 ml organ baths containing a modified Krebs-Henseleit solution of the following composition (mM): NaCl 115.3, KCl 4.6, $CaCl_2$ 1.8, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, $KH_2PO_4$ 1.1 and glucose 11.1. The medium was maintained at 31°C and aerated with 5% $CO_2$ in $O_2$. The tip of the atrial appendage was attached by a thread to a force-displacement transducer (Grass FT83C) and isometric contractions were recorded on a polygraph (Grass Model 7). Resting tension was set at 0.5 g and the preparations were allowed to equilibrate for 90 min with frequent changes of the medium. Left atria were driven by platinum electrodes delivering square wave pulses of 1 ms duration, at a frequency of 1 Hz and at a voltage slightly over threshold (0.3–1.0 V). Right atria beat spontaneously and the rate was measured by direct counting from the chart paper. Both basal tension and rate decreased initially, but there was little further change after 90 min of equilibration. Cumulative concentration-response curves for various agonists were determined before and after different $\alpha$- or $\beta$-adrenoceptor antagonists. Maximal responses were determined for each agonist and responses are expressed as the percentage of the maximal control increase in beat amplitude or rate. Absolute force and rate values are also given in some cases. Exposure to all of the receptor blocking agents was for 40 minutes. After incubation with phenoxybenzamine (PB), alone, or in combination with phentolamine or H 93/26, the preparations were repeatedly washed for at least 30 min before retesting the agonists. Phentolamine and the $\beta$-blocking agents used remained in the bath during addition of an agonist. Each preparation was used with only one blocking agent, except when the effect of a combined exposure to two blocking drugs was tested.

The validity of the use of $pD_2$ values to express agonist potency was supported by findings in preliminary experiments where neither a significant desensitization nor a significant change in maximal responses occurred when control dose-response curves were repeatedly determined in the same preparation.

### *Experiments with [$^3$H]-phenoxybenzamine*

[$^3$H]-PB hydrochloride was prepared from [$^3$H]-benzyl-chloride by the method of Nikawitz, Gump, Kerwin & Ullyot (1952), and had a specific activity of 33.13 mCi/mmol. Its physiochemical and pharmacological properties were identical to those of an authentic sample of PB hydrochloride (Smith, Kline & French), and it was shown to be radiochemically pure (Yong & Nickerson, 1973). Left atria, exposed to [$^3$H]-PB, were taken from the organ bath 2 h after removal of the drug and repeated washing of the preparation with drug-free medium. The preparations were freeze-dried, weighed, and digested in 0.5 ml NCS tissue solubilizer (Amersham/Searle) at 50°C. Counting solution (14.5 ml of Aquasol, New England Nuclear) was added and the radioactivity was measured in a liquid scintillation counter (Nuclear Chicago Mark I). Counting efficiency was assessed by the channels ratio method and a quench calibration curve, and was found to be in the range of 20 to 35%. The results are expressed as concentration of the labelled drug in the dry tissue.

### *Drugs*

The following drugs were used: (—)-arterenol bitartrate (Calbiochem; referred to as noradrenaline), (+)-isopropylnoradrenaline hydrochloride (K & K; isoprenaline), (—)-neo-synephrine hydrochloride (Sterling-Winthrop; phenylephrine), phenoxybenzamine hydrochloride (Smith, Kline & French; PB), phentolamine methanesulphonate (Ciba), (±)-propranolol hydrochloride (Ayerst) and 1-isopropylamino-3-[4-(2-methoxyethyl)-phenoxy]-2-propanol (H 93/26, Hässle). Most of the drugs were freshly diluted before each experiment in 0.9% w/v NaCl solution (saline) containing 0.01 N HCl. Stock solutions of PB and [$^3$H]-PB were made in propylene glycol containing 0.01 N HCl and were stored at −20°C. Dilutions were made with Krebs solution for each administration of the drug. Concentrations are expressed as the final concentration in M or µM in the bath.

Differences between means were evaluated by Student's two tailed $t$ test for unpaired data or the $t$ test for paired data, as appropriate, and differences with a $P$ value of 0.05 or less were considered significant.

## Results

### *Effects of altered thyroid state on adrenoceptors mediating inotropic responses*

Thyroidectomy moderately reduced and thyroid hormone treatment slightly increased the basal contractile force of left atria but the maximal developed tension in response to agonists was similar in all groups. The relative inotropic efficacies of the agonists tested were also not significantly altered by changes in thyroid state; maximal increases in force in response to noradrenaline (NA) and isoprenaline were similar and the efficacy of phenylephrine was 70 to 100% of that of the other two agonists, in any of the groups tested. However, there were significant and selective changes in the potencies of the three amines (Figure 1). Sensitivity to the relatively pure $\beta$-adrenoceptor agonist, isoprenaline, was significantly increased after thyroxine treatment and decreased following thyroidectomy, whereas sensitivity to the $\alpha$-adrenoceptor agonist, phenylephrine, was changed in

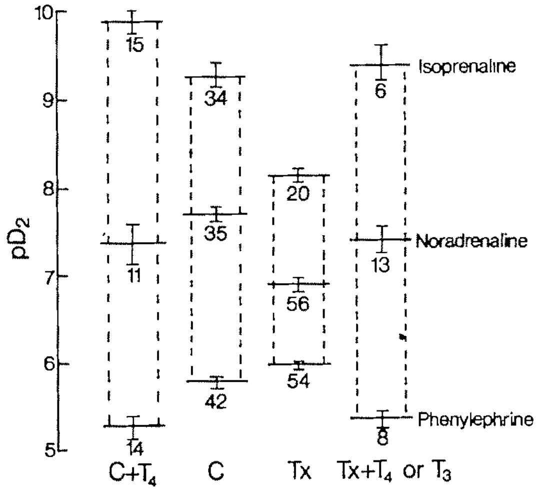

**Figure 1** Inotropic potencies of sympathomimetic agonists on left atria from rats in different thyroid states. Horizontal lines are means and vertical bars are s.e. of $pD_2$, (negative logarithm of the molar concentration that produces half-maximal response). C=control; Tx=thyroidectomized; C+$T_4$=thyroxine-treated controls; Tx+$T_4$ or $T_3$=thyroidectomized, treated with thyroid hormones. Numbers of preparations used are given by small numbers.

the opposite way. NA sensitivity was significantly reduced by thyroidectomy but was not significantly altered by thyroxine treatment. For all three amines, the change in potency observed in the hypothyroid myocardium was reversed to or beyond control levels by thyroid hormone treatment of the thyroidectomized animals. The reciprocal changes in the sensitivity of $\alpha$- and $\beta$-adrenoceptors are reflected by changes in the molar potency ratio of isoprenaline and phenylephrine. The correlation between this parameter and serum thyroxine levels in different thyroid states (see Table 1) suggests that the balance between $\alpha$- and $\beta$-adrenoceptors in the heart is under thyroid control; high thyroid hormone levels enhance the dominance of $\beta$-adrenoceptors and low hormone levels promote $\alpha$-adrenoceptor properties.

Changes in the effectiveness of $\alpha$- and $\beta$-adrenoceptor antagonists were also compatible with this possibility. The data in Table 2 demonstrate that inhibition of responses to NA by propranolol was decreased by thyroidectomy and was moderately increased after thyroxine treatment. It was also noted that the relationship between dose-ratio, and inhibitor concentration, in hypothyroid atria deviated considerably from the relation expected from simple competitive inhibition. The effect of H 93/26, a cardioselective $\beta$-blocking agent (Johansson, 1973), on inotropic responses to phenylephrine also decreased in hypothyroidism. The log dose-ratio for half-maximal responses in the presence of 0.1 μM H 93/26 and in its absence was $0.66 \pm 0.14$ in atria from thyroidectomized and $1.11 \pm 0.11$ in preparations from control animals ($P<0.05$).

In contrast to $\beta$-adrenoceptor inhibitors, the effectiveness of $\alpha$-adrenoceptor antagonists was increased after thyroidectomy and decreased following thyroid hormone treatment. Figure 2a shows that in control preparations, a 40 min exposure to 7.3 μM PB potentiated responses to NA and moderately inhibited the effect of phenylephrine. In preparations from thyroidectomized animals (Figure 2b), a similar exposure to PB partially inhibited responses to NA and blocked the effect of phenylephrine almost completely. Thyroid hormone treatment reversed the effect of PB on NA to full potentiation and reduced its blockade of responses to phenylephrine below control levels (Figure 2c). In atria from $T_4$-treated control rats (Figure 2d) PB potentiated responses to NA and did not significantly inhibit responses to phenylephrine. In association with its increased blocking effect, significantly more [$^3$H]-PB was bound to the hypothyroid myocardium ($343 \pm 18$ pmol/mg dry tissue,

**Table 2** The effect of thyroid state on the inhibitory potency of propranolol in rat left atria

| | 40 nM | Propranolol 0.4 μM | 4 μM |
|---|---|---|---|
| Control | $0.70 \pm 0.09^{a}$<br>(11) | $1.84 \pm 0.15$<br>(11) | $3.01 \pm 0.37$<br>(5) |
| Thyroidectomized | $0.37 \pm 0.07^{*}$<br>(7) | $0.70 \pm 0.10^{**}$<br>(8) | $1.39 \pm 0.07^{**}$<br>(8) |
| Thyroidectomized + $T_3$ or $T_4$ | $0.75 \pm 0.10$<br>(5) | $1.70 \pm 0.21$<br>(5) | $2.54 \pm 0.38$<br>(5) |
| Control + $T_4$ | $1.41 \pm 0.10^{**}$<br>(6) | $2.24 \pm 0.07^{*}$<br>(5) | $3.20 \pm 0.05$<br>(5) |

[a] Log dose-ratios, calculated at 50% of the maximal control inotropic response to noradrenaline. Values are means and their standard errors. Number of experiments in parentheses.

Significance of difference from corresponding control values: *$P<0.05$; **$P<0.005$.

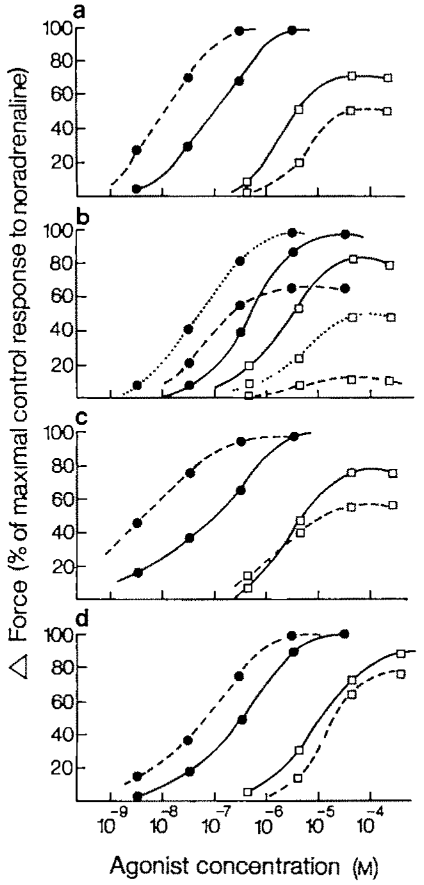

**Figure 2** Effects of phenoxybenzamine on inotropic responses to noradrenaline and phenylephrine in different thyroid states. (●) Noradrenaline; (□) phenylephrine; solid lines: control responses; dashed lines: after a 40 min exposure to phenoxybenzamine (7.3 μM); dotted lines: after exposure to phenoxybenzamine (7.3 μM) in the presence of phentolamine (26.5 μM). (a) Control preparations ($n=18$); (b) thyroidectomized ($n=21$); (c) thyroidectomized+$T_4$ or $T_3$ ($n=8$); (d) control+$T_4$ ($n=8$).

$n=22$) than to atria from either control ($262 \pm 16$, $n=13$, $P<0.005$) or from thyroidectomized, hormone-treated rats ($223 \pm 10$, $n=8$, $P<0.005$).

The increased blocking effect of PB in the hypothyroid myocardium was not similar to the 'spurious' block that can be observed when baseline contractile amplitude is increased in the presence of PB (Nickerson & Chan, 1961): after washout of the PB, the baseline contractile force was reduced slightly below control and the significant reduction in the maximal developed tension in response to NA and phenylephrine was not accompanied by a significant change in the maximal effect of $CaCl_2$ (Table 3). When hypothyroid atria were exposed to 0.7 μM PB in the presence of 12.8 μM quinidine, a drug known to reduce the rate of transmembrane $Ca^{2+}$ exchange (Silva Graça & Van Zwieten, 1972), the degree of block of responses to phenylephrine was the same as after PB alone. These observations discount the possibility that the increased blocking effect of PB in the hypothyroid myocardium is a non-specific effect on $Ca^{2+}$ movements. The change in the blocking effectiveness of PB in hypothyroidism was also observed at lower concentrations of the inhibitor (Figure 3). In the atria from control rats, 0.07 μM PB was ineffective in inhibiting responses to phenylephrine (Figure 3a) and 0.7 μM PB produced a moderate block (Figure 3c), whereas in atria from thyroidectomized animals, 0.07 μM PB produced significant inhibition (Figure 3d) and reduction of the maximal response by 0.7 μM (Figure 3d) was greater than in control preparations.

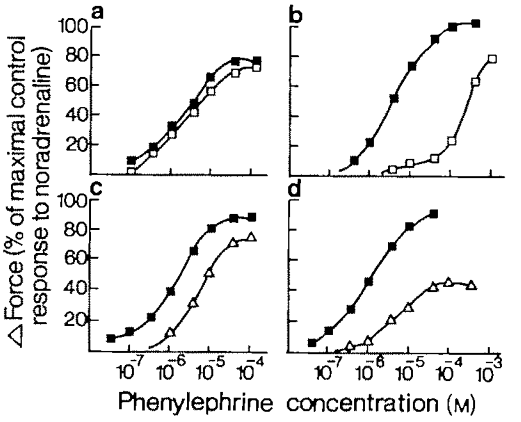

**Figure 3** Effects of low concentrations of phenoxybenzamine on inotropic responses to phenylephrine. (a) and (c) Control; (b) and (d) thyroidectomized; (■) control responses; (□) after a 40 min exposure to phenoxybenzamine (0.07 μM); (Δ) after exposure to phenoxybenzamine (0.7 μM). Number of experiments: 4 (a), 3 (b), 4 (c), 7 (d).

Thyroidectomy also increased the blocking effect of the reversible α-adrenoceptor antagonist, phentolamine. The marginal inhibition by 2.6 μM phentolamine of responses of control atria to phenylephrine (log dose-ratio $0.32 \pm 0.09$, $n=5$) was significantly increased in preparations from thyroidectomized rats ($0.80 \pm 0.11$, $n=5$, $P<0.01$). Inhibition by phentolamine was reversible by washing the preparations for 30 to 60 minutes. When left atria, from thyroidectomized animals, were exposed to 7.3 μM [$^3$H]-PB in the presence of 26.5 μM phentolamine and then washed for at least an hour (6

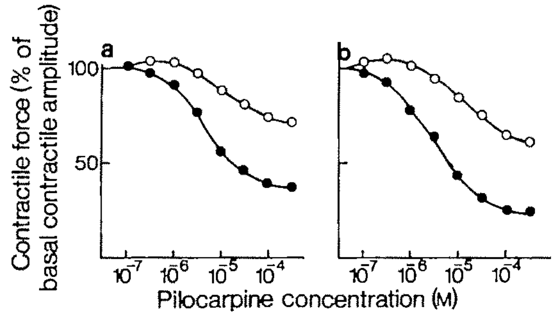

**Figure 4** Inhibition of phenoxybenzamine of the negative inotropic response to pilocarpine. (a) Control ($n=6$); (b) thyroidectomized ($n=8$). (●) Control responses, (○) after a 40 min exposure to phenoxybenzamine (0.7 μM).

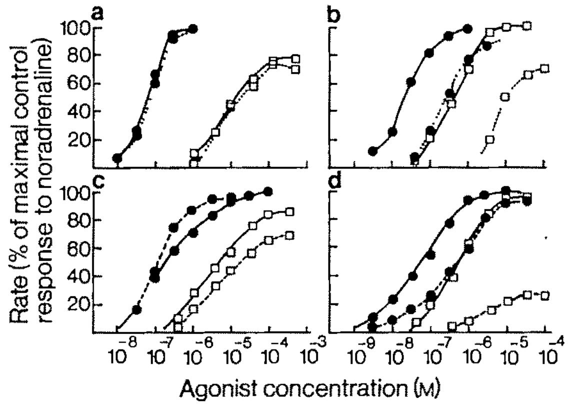

**Figure 5** The effect of combined exposure to $\beta$- and $\alpha$-adrenoceptor blocking drugs on inotropic responses to sympathomimetic drugs in different thyroid states. (a) and (c) Control; (b) and (d) thyroidectomized. (●) Noradrenaline; (□) phenylephrine. Solid lines: control responses; dotted lines (a, b): after a 40 min exposure to phenoxybenzamine (0.007 μM) in the presence of H 93/26 (0.1 μM); dashed lines (c, d): after exposure to phenoxybenzamine (0.07 μM) in the presence of H 93/26 (0.1 μM). Numbers of experiments: 4 (a), 5 (b), 5 (c), 5 (d). For explanation see text.

experiments), protection of $\alpha$-adrenoceptors was shown by a significant reduction in both the block by (Figure 2b, dotted lines) and in the retention of [$^3$H]-PB (from 343 ± 18 to 247 ± 16 pmol/mg dry tissue, $P<0.005$). In 6 control preparations, a similar combination of the two antagonists resulted in potentiation of responses to NA identical to that caused by PB alone, and retention of [$^3$H]-PB was the same (265 ± 17) as after exposure to [$^3$H]-PB alone (262 ± 16 pmol/mg dry tissue).

In the presence of phentolamine, potentiation by PB of responses of hypothyroid atria to NA (Figure 2b) was not different from the potentiation seen in control preparations (Figure 2a), indicating that this effect was not influenced by the change in thyroid state. Inhibition by PB of the negative inotropic effect of pilocarpine was also not affected by thyroidectomy (Figure 4). In the latter experiments, pilocarpine was used as an agonist, since the use of acetylcholine for quantitative evaluation of cholinoceptor blockade in tissues with high cholinesterase activity is misleading.

These observations suggest that the altered reactivity of the hypothyroid tissue to PB is selective to $\alpha$-adrenoceptors.

Since $\beta$-adrenoceptor activity was only reduced but not abolished after thyroidectomy, the effect of $\alpha$-adrenoceptor antagonists could have been masked by simultaneous activation of $\beta$-adrenoceptors by agonists. To reduce the $\beta$-adrenergic component of inotropic reactions, some preparations were exposed to PB in the presence of a small concentration of a $\beta$-adrenoceptor antagonist (Figure 5). The effect of

**Table 3** The effect of phenoxybenzamine (PB) on basal contractile force and on maximal contractile responses to agonists in left atria from thyroidectomized rats

| | *Baseline contractile force (mg)* | *Maximal increase in contractile force (mg)* | | |
|---|---|---|---|---|
| | | *NA* | *Phenylephrine* | *CaCl$_2$* |
| Control | 341 ± 106 | 743 ± 167 | 537 ± 85 | 623 ± 137 |
| After PB 0.7 μM | 326 ± 101 | 705 ± 170 | 250 ± 101 | 697 ± 141 |
| % change | −4.2 ± 3.2 | −5.9 ± 3.9 | −54.7 ± 7.4* | +13.0 ± 5.1 |
| Control | 293 ± 52 | 660 ± 92 | 538 ± 76 | 514 ± 90 |
| After PB 7.3 μM | 271 ± 36 | 583 ± 107 | 84 ± 21 | 537 ± 85 |
| % change | −0.6 ± 4.8 | −15.0 ± 5.6* | −81.8 ± 4.7** | +0.3 ± 4.1 |

Significant change: *$P<0.05$; **$P<0.005$
Number of experiments is 6 (7.3 μM PB) and 3 (0.7 μM PB).

0.1 μM H 93/26 on responses to phenylephrine was first tested and the preparations were then washed for an hour. This time was sufficient to reverse the observed inhibition almost completely. As in other experiments mentioned above, inhibition was significantly greater in control preparations than in atria from thyroidectomized animals. The preparations were then incubated with the same concentration of H 93/26 and, 10 min later, PB (0.007 or 0.07 μM) was added to the medium. After a 40 min incubation with both drugs, the preparations were washed for an hour with drug-free medium and the effects of the agonists were retested. The results show that in control preparations, simultaneous inhibition of $\beta$-adrenoceptors did not significantly enhance the degree of $\alpha$-adrenoceptor blockade: low concentrations of PB remained practically ineffective in inhibiting responses to either NA or phenylephrine (Figure 5a,c). On the other hand, in atria from thyroidectomized animals the observed inhibition was clearly greater than after PB alone. Exposure to 0.07 μM PB in the presence of 0.1 μM H 93/26 inhibited the effects of NA and blocked responses to phenylephrine almost completely (Figure 5d, compare to results in Figure 3b), and even 0.007 μM PB produced significant inhibition of responses to both agonists (Figure 5b). These experiments indicate that in control preparations the strong dominance of $\beta$-adrenoceptors is not significantly reduced after their moderate inhibition, whereas in atria from thyroidectomized rats, an even smaller inhibition produced by the same concentration of the $\beta$-blocking drug, significantly enhanced the apparent $\alpha$-adrenoceptor activity.

### *The effect of altered thyroid state on adrenoceptors mediating chronotropic responses*

Although changes in thyroid hormone levels caused significant and predictable changes in basal right atrial rate (Table 1), the maximal increase in rate produced by agonists and their relative efficacies were not significantly altered. The maximal increase in heart rate produced by NA was slightly less than that after isoprenaline and slightly more than that after phenylephrine in all four groups. However, there were selective changes in the potencies of agonists, qualitatively similar to those observed with inotropic reactions in left atria. The data in Table 4 show that thyroidectomy reduced the potency of isoprenaline and increased the potency of phenylephrine, which resulted in a decrease in their molar potency ratio. Unlike inotropic responses, this change was not fully reversed by thyroid hormone treatment and thyroxine treatment of control rats failed to alter significantly the chronotropic potency of agonists. The same trend could be observed when inhibition by propranolol of chronotropic responses to NA was determined in different thyroid states (Table 5). The significant decrease in the effectiveness of propranolol in hypothyroidism was only partially reversed by thyroid hormone treatment and the increase in the inhibitory effect of propranolol in thyroxine-treated control rats was not statistically significant. The conclusion from these experiments, that chronotropic responses of control atria are closer to maximal $\beta$-adrenoceptor dominance than inotropic responses, was corroborated by results obtained with $\alpha$-adrenoceptor antagonists. In contrast to inotropic responses, chronotropic responses to phenylephrine did not have a significant $\alpha$-adrenoceptor component: PB (Figure 6a) or phentolamine (Figure 6c) failed to inhibit the increase in heart rate produced by phenylephrine. In right atria from thyroidectomized animals, the appearance of $\alpha$-adrenoceptors mediating chronotropic responses was indicated by the significant inhibition of the effect of phenylephrine by PB (Figure 6b) or by phentolamine (Figure 6d).

Changes in thyroid state were associated with significant changes in the slopes of concentration-response curves for chronotropic effects. Changes in

**Table 4** Chronotropic potency of adrenoceptor agonists in different thyroid states

| | *Isoprenaline* | *Noradrenaline* | *Phenylephrine* |
|---|---|---|---|
| Control | 9.49 ± 0.21<br>(11) | 7.84 ± 0.11<br>(17) | 5.64 ± 0.09<br>(16) |
| Thyroidectomized | 8.68 ± 0.23*<br>(15) | 6.83 ± 0.12**<br>(29) | 6.14 ± 0.16*<br>(12) |
| Thyroidectomized + $T_3$ or $T_4$ | 9.05 ± 0.10<br>(5) | 7.18 ± 0.16**<br>(15) | 5.25 ± 0.21<br>(7) |
| Control + $T_4$ | 9.40 ± 0.14<br>(7) | 8.09 ± 0.16<br>(8) | 5.76 ± 0.14<br>(5) |

Values shown are means ± s.e. of $pD_2$ (negative logarithm of molar concentration required to produce half-maximal response). Numbers of experiments are given in parentheses.
Significance of difference from corresponding value in controls: $^*P < 0.05$; $^{**}P < 0.005$.

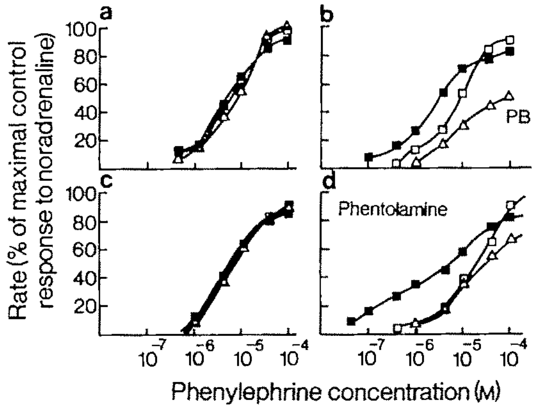

**Figure 6** Effects of $\alpha$-adrenoceptor antagonists on chronotropic responses to phenylephrine in different thyroid states. (a) and (c) Control; (b) and (d) thyroidectomized. (■) Control responses; (□) after a 40 min exposure to 0.7 µM phenoxybenzamine (a and b) or to 0.3 µM phentolamine (c and d); (△) after a 40 min exposure to 7.3 µM phenoxybenzamine (a and b) or 2.6 µM phentolamine (c and d). Number of experiments: 4 (a), 4 (b), 6 (c), 7 (d).

the slopes of inotropic concentration-response curves were smaller and less consistent. Although the slope of the concentration-response relationship in a complex system can be influenced by a variety of factors and its value in studying quantitative aspects of drug-receptor interactions is limited, the parallel between the observed changes in receptor properties and changes in slopes of the concentration-response curves of chronotropic reactions justifies a limited quantitative analysis of dose-response relationships. A simple way to describe concentration-response curves is to use the ratio of two concentrations causing specified effects (Stephenson, 1956). Table 6 shows the logarithms of the ratios of concentrations that produce 50% and 20% of the maximal chronotropic responses to isoprenaline and to phenylephrine. In control preparations, where both agonists exerted their effects on $\beta$-adrenoceptors, steep slopes resulted in small log-dose ratios: the ratio was smaller for isoprenaline than for phenylephrine and both were smaller than the value of 0.60 (log of 4) predicted by a 1 : 1 or non-cooperative drug-receptor interaction. In right atria from thyroidectomized animals, the appearance of an $\alpha$-adrenoceptor component in the chronotropic effect of phenylephrine was associated with a significant decrease in slope, indicated by the increase in ratio. The increase in ratio for isoprenaline was much smaller. Inhibition of $\alpha$-adrenoceptors by phentolamine or a low concentration of PB decreased the log-ratio toward that in control preparations as also illustrated by the steeper concentration-response curves in Figure 6b and d whereas inhibition of $\beta$-adrenoceptors by H 93/26 produced a further increase in ratio, i.e. decrease in slope. These observations suggest that activation of myocardial $\beta$-adrenoceptors result in steeper dose-response curves than activation of $\alpha$-adrenoceptors, and a change in the balance of $\alpha$- and $\beta$-adrenoceptors results in a corresponding change in slopes.

## Discussion

The results presented establish a strong correlation between thyroid hormone levels and the relative importance of myocardial $\alpha$- and $\beta$-adrenoceptors mediating inotropic and chronotropic responses. In general, thyroidectomy shifted the balance of adrenoceptors toward the dominance of the $\alpha$ component, whereas maximal dominance of $\beta$-adrenoceptors was observed in preparations from thyroxine-treated animals. The properties of adrenoceptors in the myocardium of euthyroid rats appeared to be closer to those in hyperthyroid than in hypothyroid animals.

The observed shifts in the balance between the two kinds of receptors can be interpreted as due to changes in the equilibrium between two forms of a single type

**Table 5** Inhibition of chronotropic responses to noradrenaline by propranolol in different thyroid states

| | *40 nM* | *Propranolol 0.4 µM* | *4 µM* |
|---|---|---|---|
| Control | 1.43±0.17[a]<br>(4) | 2.15±0.23<br>(4) | 3.09±0.27<br>(4) |
| Thyroidectomized | 0.84±0.19*<br>(9) | 1.28±0.25*<br>(10) | 1.86±0.29*<br>(10) |
| Thyroidectomized<br>+$T_3$ or $T_4$ | 1.07±0.20<br>(8) | 1.78±0.22<br>(8) | 3.07±0.31<br>(8) |
| Control+$T_4$ | 1.76±0.14<br>(4) | 2.51±0.18<br>(4) | 3.38±0.24<br>(4) |

[a] Log dose-ratios, calculated at 50% of the maximal chronotropic response to noradrenaline. Values are means and their standard errors. Number of experiments is given in parentheses.
Significance of difference from corresponding control values: $^*P<0.05$.

of unit, as proposed earlier (Kunos & Szentiványi, 1968; Kunos & Nickerson, 1976), although they can also be explained by two independent pools of receptors with reciprocal sensitivities to the effects of thyroid hormones. In earlier observations on temperature-induced receptor transformation the first interpretation was favoured by the finding that $\beta$-adrenoceptors did not appear at high temperatures after $\alpha$-adrenoceptors had been alkylated at a low temperature (Kunos *et al.*, 1973; Kunos & Nickerson, 1976), and there are analogies between the two models that suggest that the changes in responsiveness produced by altered thyroid hormone levels reflect a similar interconversion of myocardial $\alpha$- and $\beta$-adrenoceptors.

The metabolic rate of tissues is low both in hypothyroidism and at low temperatures. Similarly to the effect of hypothyroidism in rats, low temperature in frog hearts induced reciprocal changes in the effectiveness of $\alpha$- and $\beta$-blocking agents (Kunos & Szentiványi, 1968; Buckley & Jordan, 1970; Harri, 1973; Kunos & Nickerson, 1976), significantly reduced the potency ratio of isoprenaline and phenylephrine (Harri, 1973; Tirri, Harri & Laitinen, 1974; Kunos, unpublished), and increased the tissue binding of [$^3$H]-PB (Kunos & Nickerson, 1976). The $\alpha$-adrenoceptor component in inotropic responses appears to require intact sympathetic innervation and denervation of rats by 6-hydroxydopamine was shown to stabilize the receptor in the $\beta$-form both at low temperature (Kunos *et al.*, 1973) and in hypothyroidism (Kunos & Mucci, 1975). Both hypothyroidism and low temperature decreased the sensitivity of the myocardium to the natural transmitter adrenaline (frog) or NA (rat), which suggests that $\alpha$- and $\beta$-adrenoceptors may be low- and high-sensitivity forms of the same unit. Adrenoceptor reactivity in frog isolated hearts shows seasonal variations and the greater sensitivity of the winter frog heart to $\alpha$-adrenoceptor blockade could be abolished by treating frogs with anterior pituitary extract, which possibly increased thyroid activity (Nickerson & Nomaguchi, 1950), or by raising the temperature of the isolated heart (Kunos & Szentiványi, 1968). These findings and our unpublished observation that the shift in the properties of myocardial adrenoceptors after thyroidectomy was not significantly increased by lowering the temperature of these preparations, suggest that the mechanisms by which thyroid hormones and temperature can influence receptors are similar.

Although adrenoceptors mediating inotropic and chronotropic responses were affected by changes in thyroid hormone levels in a qualitatively similar way, the equilibrium between the $\alpha$ and the $\beta$ component was different for the two reactions. In contrast to inotropic responses, and in agreement with published observations (Krell & Patil, 1969; Parr & Urquilla, 1972), chronotropic responses of control atria did not have an $\alpha$-adrenoceptor component. This difference was also apparent in preparations from thyroidectomized rats, where propranolol was more effective and PB was less effective in inhibiting chronotropic than inotropic responses. Maximal dominance of $\beta$-adrenoceptors in chronotropic responses of control atria may explain why the potency of agonists for this effect did not significantly change after thyroxine treatment. These observations indicate that the equilibrium between $\alpha$- and $\beta$-adrenoceptors is different for different kinds of responses. Observations on the effects of thyroid state on myosin ATP-ase activity show important species differences in whether conditions in the euthyroid animal are closer to those in the hypothyroid or in the hyperthyroid state (Yazaki & Raben, 1975). Similar differences may exist for receptor equilibrium and may have contributed to the considerable controversy in the literature on the effects of altered thyroid states on

**Table 6** Slopes of chronotropic concentration-response curves in different thyroid states

| *Agonist:*<br>*Antagonist:* | *Isoprenaline*<br>— | <br>*Phenoxybenzamine, 0.7 μM or phentolamine, 0.3 μM* | | *Phenylephrine*<br>— | | <br>*H 93/26, 0.1 μM* |
|---|---|---|---|---|---|---|
| Control | $0.45 \pm 0.02$<br>(13) | — | | $0.55 \pm 0.04$<br>(15) | | — |
| Thyroidectomized | $0.60 \pm 0.06$<br>(11) | $0.55 \pm 0.05$<br>(7) | $P < 0.05$ | $0.84 \pm 0.09$<br>(18) | $P > 0.05$ | $1.26 \pm 0.27$<br>(5) |
| *P* value | $< 0.05$ | | | $< 0.01$ | | |

Values are means ± s.e. of log dose-ratio (logarithm of the ratio of $EC_{80}$ and $EC_{20}$ for isoprenaline or for phenylephrine). Numbers of experiments are given in parentheses.

catecholamine sensitivity (Harrison, 1964; Waldstein, 1966). Another source of discrepancies may be the inconsistency in the way sensitivity to agonists has been determined; although the conclusion in some recent studies was a lack of change in myocardial sensitivity to noradrenaline in altered thyroid states (Buccino, Spann, Pool, Sonnenblick & Braunwald, 1967; Levey, Skelton & Epstein, 1969), changes similar to those in the present study are apparent when concentrations of noradrenaline producing half-maximal responses are determined from the data presented by these authors.

The specificity of $\alpha$-adrenoceptor blockade in the present study is supported by several observations. Thyroidectomy increased the blocking effectiveness of both PB and phentolamine, antagonists with very different chemical structures and reactivities. PB is known to have a wide range of effects other than inhibition of $\alpha$-adrenoceptors, which probably all involve alkylation of various tissue components (Harvey & Nickerson, 1954). However, potentiation of responses to noradrenaline after protection of $\alpha$-adrenoceptors by phentolamine (Figure 2b) and inhibition of the negative inotropic effect of pilocarpine by PB (Figure 4) were unaffected by thyroidectomy, which indicated a selective increase in the interaction of PB with $\alpha$-adrenoceptors. The absence of a significant change in the reactivity of PB with nonspecific tissue components is also suggested by the finding that after protection of $\alpha$-adrenoceptors by phentolamine, binding of [$^3$H]-PB was reduced to a level not significantly different from that in controls. PB also did not influence the inotropic response to $CaCl_2$ and its inhibition of responses to phenylephrine was not altered by the presence of quinidine, which suggested that the effect of PB on $\alpha$-adrenergic responses was not related to a nonspecific inhibition of transmembrane $Ca^{2+}$ movements that can occur with a concentration of PB (0.1 $\mu$M) much higher than those used in the present study (Shibata, Carrier & Frankenheim, 1968).

The mechanism by which threshold blocking concentrations of a $\beta$-adrenoceptor antagonist during exposure to PB significantly potentiate the apparent blocking effect of the latter in hypothyroid preparations (Figure 5) or in frog hearts at low temperatures (unpublished) is not readily explained. Persistence of the increased blocking effect of PB, after the $\beta$-blocking drug had been washed out, suggests that this potentiation is not due to an 'occult' block of $\alpha$-adrenoceptors, masked by activation of $\beta$-adrenoceptors. If this were so, potentiation of the blocking effect of PB should have been reversed after removal of the $\beta$-blocking drug, but it was not. Instead, it may be proposed that inhibition of the $\beta$ form of the receptor shifted the equilibrium in favour of the $\alpha$ form, which was then 'trapped' by the irreversible antagonist. This effect would be similar to the shift in equilibrium, induced by antagonists, between conformational states of an allosteric cholinoceptor (Karlin, 1967). The above mechanism might also account for observations that in euthyroid preparations PB can increase the inhibition of inotropic responses to NA and phenylephrine by a high concentration of pronethalol or propranolol, if given after but not before the $\beta$-blocking drug (Govier, 1968; Kaumann, 1970). It is also interesting to note that changes in the relative dominance of $\alpha$- and $\beta$-adrenoceptor responses in the present study were associated with changes in the slopes of agonist concentration-response curves, although the mechanism underlying these changes is not clear.

Observations of a temperature-induced interconversion of histamine $H_1$- and $H_2$-receptors in guinea-pig ileum (Kenakin, Kreuger & Cook, 1974) and of a thyroid hormone-dependent change in the concentration of œstrogen receptors in the rat pituitary gland (Cidlowski & Muldoon, 1975) may suggest that metabolic control of receptors may be a general phenomenon affecting not only adrenoceptors, but other hormone receptors as well.

The present findings on the pharmacological activity of drugs acting on adrenoceptors in preparations from thyroidectomized animals are in good agreement with previous reports of an increased effectiveness of phenylephrine and phentolamine and decreased effectiveness of isoprenaline and propranolol in hearts from propylthiouracyl-fed rats (Nakashima, Maeda, Sekiya & Hagino, 1971; Nakashima & Hagino, 1972). The observation of an increased binding of [$^3$H]-PB to the hypothyroid myocardium in the present study suggests that the change in receptor response is due to a change in the number of available binding sites. Although PB can bind to a number of nonspecific tissue components, the parallel decrease in block and binding after protection of $\alpha$-adrenoceptors by phentolamine, *in vitro*, or thyroid hormone treatment *in vivo*, indicates that the difference in the binding of PB is associated with $\alpha$-receptors and that its nonspecific binding is not affected by changes in thyroid state.

Interconversion of $\alpha$- and $\beta$-adrenoceptors due to altered thyroid hormone levels could account for a number of other published observations. A selective increase in adrenergic stimulation of the aorta of hypothyroid rabbits (Rosenquist & Boréus, 1972), a diminished calorigenic response to noradrenaline in thyroid hormone deficient newborn rats (Steele & Wekstein, 1973) and a decreased glycogenolytic response to isoprenaline in $^{131}$I-treated rats (Dominguez, Catanzaro, Fernandez & Vidal, 1973) suggest that the hormone-induced change in adrenoceptor properties may not be limited to the myocardium. Other observations, including a selective increase in isoprenaline sensitivity of cultured mouse heart cells exposed to triiodothyronine (Wildenthal, 1974) and a decreased adrenergic vasoconstriction, reversed by pronethalol, in the hind limb of thyroxine-treated dogs (Zsoter, Tom & Chappel, 1964) are compatible with a shift in receptor properties from $\alpha$ to $\beta$ in hyperthyroidism. Thyroid hormones may also modulate their own release by influencing the properties of adrenoceptors located on cells of the thyroid gland; isoprenaline was shown to increase the *in vitro* synthesis of thyroid hormones in isolated calf thyrocytes, an effect blocked by phentolamine, or increase the *in vivo* secretion of thyroid hormones in thyroxin pretreated mice, an effect blocked by propranolol (cited by Melander, Ranklev, Sundler & Westgren, 1975).

Although inotropic and chronotropic responses of the myocardium can be mediated by both $\alpha$- and $\beta$-adrenoceptors, the underlying mechanisms may differ. Stimulation of $\beta$-adrenoceptors is associated with a rise in cyclic AMP levels both in intact hearts (Robison, Butcher, Øye, Morgan & Sutherland, 1965) and in subcellular membrane preparations (Murad, Chi, Rall & Sutherland, 1962). Preliminary observations in our laboratory showed that $\beta$-adrenoceptor stimulation increased cyclic AMP levels both in contracting and in quiescent myocardial preparations, whereas an $\alpha$-adrenoceptor mediated elevation of cyclic AMP levels in hypothyroid left atria required not only intact cells but contractile activity as well (Kunos, 1975). It has also been reported that stimulation of $\alpha$-adrenoceptors in the dog heart is not associated with an 'oxygen-wasting' effect (Privitera & Rosenblum, 1967) and it is interesting to note that both low temperature in frogs (Korb, Poupa & Carlsten, 1973) and hypothyroidism in rats (Chappel, Rona & Gaudry, 1959) appear to prevent isoprenaline-induced myocardial necrosis. Therefore, it is tempting to speculate that changes in adrenoceptor characteristics play a role in the adaptation of mechanical responses of the myocardium to its metabolic state. Such as adaptation might contribute to the reported therapeutic effect of lowering thyroid activity in patients with angina pectoris (Segal, Silver, Yohalem & Newburger, 1958) and to the increased sensitivity of the hyperthyroid and decreased sensitivity of the hypothyroid myocardium to arrhythmias (Freedberg, Papp & Vaughan Williams, 1970).

Propranolol was the gift of Ayerst Laboratories and H 93/26 was kindly provided by Dr E. Carlsson (Hässle Laboratories). I thank Dr Man Sen Yong for synthesis of the [$^3$H]-phenoxybenzamine used in this study and Dr M. Nickerson for reading the manuscript. The work was supported by grants-in-aid from the Medical Research Council of Canada.

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*(Received June 30, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 191–199

# PRODUCTION OF PROSTAGLANDINS BY THE PSEUDOPREGNANT RAT UTERUS, *in vitro,* AND THE EFFECT OF TAMOXIFEN WITH THE IDENTIFICATION OF 6-KETO-PROSTAGLANDIN $F_{1\alpha}$ AS A MAJOR PRODUCT

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1 Prostaglandin production by rat uterus homogenates has been studied, *in vitro*, on days 2 to 13 of pseudopregnancy.

2 The highest production of prostaglandins occurred on day 5.

3 The amounts of prostaglandins F and D formed were higher than the amounts of prostaglandin E on every day studied.

4 The ratios of prostaglandins F and D to prostaglandin E produced steadily decreased up to day 6. It then increased with the highest values occurring between days 10 and 13.

5 Progesterone levels in peripheral plasma increased rapidly from days 2 to 5, remained high up to day 9, then steadily decreased between days 10 and 13.

6 The anti-oestrogenic drug, tamoxifen administered on day 2, significantly inhibited the increase of prostaglandin production which occurred on day 5. Prostaglandin E production was inhibited more than the production of prostaglandins F and D.

7 Analysis of the uterine extracts by gas chromatography and mass spectrometry showed prostaglandin $F_{2\alpha}$, $F_{1\alpha}$ (in trace amounts), $E_2$ and $D_2$ to be present.

8 The major product detected was 6-keto-prostaglandin $F_{1\alpha}$. Its identification forms an addendum to the paper.

9 Also present as a major product was 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid.

## Introduction

Oestradiol treatment of ovariectomized rats increases the prostaglandin $F_{2\alpha}$ synthesizing capacity of the uterus *in vitro* by increasing the amount of prostaglandin synthetase present (Ham, Cirillo, Zanetti & Kuehl, 1975). Oestradiol treatment of ovariectomized guinea-pigs produces similar results (Naylor & Poyser, 1975) and the action of the oestradiol is not affected by the simultaneous administration of progesterone. During the oestrous cycle of the guinea-pig, the prostaglandin $F_{2\alpha}$ synthesizing capacity of the uterus increases towards the end of the cycle (Poyser, 1972) and prostaglandin $F_{2\alpha}$ levels in the uterine venous plasma rise (Blatchley, Donovan, Horton & Poyser, 1972; Earthy, Bishop & Flack, 1975). Oestradiol output from the ovary increases during this period (Joshi, Watson & Labhsetwar, 1973; Blatchley, Maule, Walker & Poyser, 1975), so it is possible that oestradiol is the physiological stimulus for increased synthesis and release of prostaglandin $F_{2\alpha}$ from the uterus.

There is much clinical interest at present in drugs which inhibit prostaglandin synthesis. The non-steroidal, anti-inflammatory drugs inhibit prostaglandin synthetase (Vane, 1971) and thereby prevent the conversion of the precursor fatty acids into prostaglandins. Prostaglandin synthetase is apparently inactivated during the formation of prostaglandins (Lands, LeTellier & Vanderhoek, 1973), so the continual synthesis of new enzyme would appear a critical factor in determining the amounts of prostaglandins formed by a tissue. If the levels of prostaglandin synthetase in certain tissues are under hormonal control, drugs which inhibit the action and/or release of the hormone should maintain low levels of enzyme in the tissue and thereby reduce the prostaglandin synthesizing capacity of that tissue. Such drugs may be of clinical importance in inhibiting prostaglandin synthesis.

In this study, we have tested the effect of the anti-oestrogen, tamoxifen (Harper & Walpole, 1967) on the prostaglandin synthesizing capacity, *in vitro*, of the pseudopregnant rat uterus. Tamoxifen is an anti-oestrogenic drug in the rat, antagonizing the effects of circulating oestrogen at the tissue level (Harper & Walpole, 1967). It has also been shown to reduce the output of oestradiol from the rat ovary, either by directly inhibiting the ovarian synthesis of oestradiol, or indirectly by blocking follicle stimulating hormone release from the pituitary (Watson, Anderson, Alum, O'Grady & Heald, 1975). Plasma oestradiol levels in the pseudopregnant rat show a small rise up to day 4 (though the peak level may be late on day 3), fall to a lower level which is maintained up to about day 9 or 10, then increase rapidly over the next 3 days (Welschen, Osman, Dullaart, De Greef, Uilenbroek & De Jong, 1975). Initially, we had to study how much prostaglandin was produced normally by the rat uterus on each day of pseudopregnancy. This is of interest also since prostaglandin $F_{2\alpha}$ released from the rat uterus may be the endogenous substance which terminates pseudopregnancy in the rat (Saksena, Watson, Lau & Shaikh, 1974; Weems, Pexton, Butcher & Inskeep, 1975; Castracane & Shaikh, 1976).

## Methods

Female Wistar rats were housed in conditions of 12 h day and 12 h night, with the light changes occurring at 8 h 00 min and 20 h 00 min respectively. They received food and water *ad libitum*. Vaginal smears were taken daily and examined microscopically. Day 1 of the oestrous cycle was taken as the day of maximum cornification of the smear, preceding the day of leucocytic infiltration. All rats used had exhibited regular 4 day cycles. Pseudopregnancy was achieved by mating the female rats with a vasectomized male. Mating was assumed to have taken place during the dark period between day 4 and day 1. This latter day now became day 1 of pseudopregnancy. All rats, except two, became pseudopregnant at the expected time as assessed by the lack of a subsequent oestrus and/or by the determination of peripheral plasma progesterone levels. The pseudopregnant rats were used in the following two experiments.

### *Prostaglandin production by pseudopregnant rat uterus* in vitro

Four or five rats per day were killed on the mornings of days 2 to 13 of pseudopregnancy. Each rat was lightly anaesthetized with ether and a 2 ml sample of blood withdrawn from the heart into a heparinized syringe. The blood was centrifuged, the plasma withdrawn and stored at −20°C. Each rat was killed by incising the neck. The uterus was removed, weighed, homogenized and incubated for 90 min in the same manner as described previously for the guinea-pig uterus (Poyser, 1972). The pH of the incubate was then lowered to 4.5 with 1 N HCl and the prostaglandins present extracted with re-distilled ethyl acetate. The ethyl acetate extract was evaporated to dryness on a rotary evaporator at 45°C, and the residue dissolved in 10 ml re-distilled ethyl acetate and stored at −20°C. The amounts of prostaglandins isolated were measured by radioimmunoassay using antisera produced against prostaglandin $F_{2\alpha}$ and $E_2$. The technique used was identical to that employed for assaying prostaglandins in ethyl acetate extracts of guinea-pig uterus (Mitchell, Poyser & Wilson, 1976). One further cross-reactivity study was performed, using prostaglandin $D_2$ against the prostaglandin $E_2$ antibody. Its cross-reactivity was very low (0.003%). Prostaglandin $D_2$ cross-reacts 17.1% with the $F_{2\alpha}$ antibody.

Progesterone was measured in the peripheral plasma samples of all rats by radioimmunoassay in the manner described previously (Poyser & Horton, 1975), though the plasma usually had to be diluted 10 times with distilled water before assay. The values obtained were in the expected range (Welschen *et al.*, 1975), though marginally lower.

### *Effect of tamoxifen on prostaglandin production by the pseudopregnant rat uterus* in vitro

This study was performed following the results obtained from the first experiment, and in light of the reported finding that tamoxifen administered on day 2 to pregnant rats inhibits the increased output of oestradiol from the ovary which normally takes place on days 3 and 4 (Watson *et al.*, 1975). Five rats were injected subcutaneously at 11 h 00 min on day 2 of pseudopregnancy with 0.05 mg tamoxifen (approx. 0.25 mg/kg). The drug was provided absorbed on to mannitol (1%) and was dissolved in 0.5% aqueous Tween 80 so that 0.5 ml contained 0.05 mg tamoxifen. Five control rats were injected with 0.5 ml aqueous Tween 80 at the same time on day 2. All rats were killed on the morning of day 5. Peripheral plasma levels of progesterone and prostaglandin production by uterine incubates were measured by radioimmunoassay, as in the first experiment.

### *Identification of prostaglandins*

To facilitate the handling and identification of prostaglandins, samples from several days were pooled together, namely days 2, 4 and 5 (from the first experiment), days 7 and 9, half the samples from each of days 10, 11 and 12, and the samples from the second experiment. This gave 5 pooled samples. (The specific and more detailed analysis of the remaining samples forms an addendum to this paper).

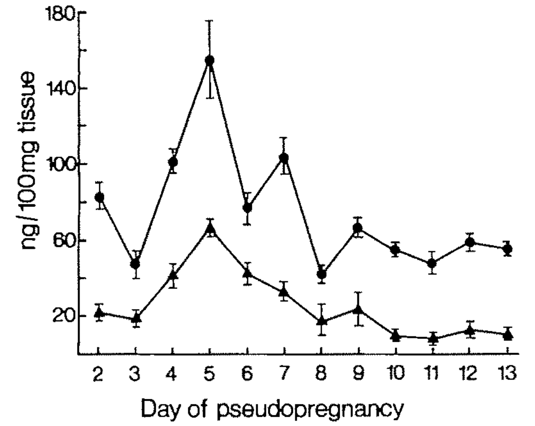

**Figure 1** Production of prostaglandins F and D (●) and prostaglandin E (▲) by homogenates of pseudopregnant rat uteri incubated *in vitro*. (See Table 1 for number of animals used per day); s.e. means represent 'between rat' variations.

Each pooled sample was evaporated to dryness and the residue dissolved in 20 ml 67% ethanol. This solution was washed twice with 20 ml petroleum ether (b.p. 60° to 80°C), and then evaporated to dryness. The extract was further purified by silicic acid column chromatography performed as described by Blatchley *et al.* (1972).

Prostaglandins present in the 'F' fraction from the column were converted into the corresponding methyl ester trimethylsilyl ethers (Me-TMS). (Blatchley *et al.*, 1972). The Me-TMS derivatives of authentic prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ were prepared for comparative purposes when the extracts were analysed by combined gas chromatography and mass spectrometry (GC-MS).

Prostaglandins present in the 'E' fraction from the column were converted to the methyl esters by reacting with diazomethane for 15 minutes. The *n*-butyloximes were then prepared by reacting the methyl esters with O-*n*-butylhydroxylamine hydrochloride (5 mg/ml in dry pyridine) for 90 min at 60°C. Finally the trimethylsilyl ethers were prepared by reacting the methyl *n*-butyloximes with 20 µl *N,N*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) at 60°C for 15 minutes. The same derivatives of prostaglandin $E_2$, $E_1$, $D_2$ and 13,14,dihydro-15,keto-prostaglandin $F_{2\alpha}$ were also prepared for comparative purposes when the extracted samples were analysed by GC-MS.

## Results

### *Prostaglandin production by the pseudopregnant rat uterus* in vitro

The results are shown in Figure 1 and the amounts of prostaglandins produced are expressed as ng prostaglandin per 100 mg tissue. Levels of prostaglandins F and D were significantly higher on day 2 than on day 3 ($P<0.01$). Levels rose significantly on day 4 ($P<0.01$) and again on day 5 ($P<0.05$), to reach the highest value seen on any day of pseudopregnancy. Levels fell significantly on day 6 ($P<0.01$), rose significantly on day 7 ($P<0.05$), fell significantly on day 8 ($P<0.01$), and rose significantly again on day 9 ($P<0.01$) to remain relatively stable, with small fluctuations, up to the end of pseudopregnancy.

Prostaglandin E levels again showed a peak at day 5, but did not show the same fluctuations from day to day as did the levels of prostaglandins F and D. The levels of prostaglandin E were similar on days 2 and 3, rose significantly on day 4 ($P<0.05$) and again on day

**Table 1** Ratio of prostaglandin F and D (PGF and D) to prostaglandin E (PGE) in uterine incubates and peripheral plasma levels of progesterone during pseudopregnancy in the rat

| *Day of pseudopregnancy* | *No. of rats (n)* | *Ratio of PGF and D to PGE* | *Progesterone level ng/ml (mean ± s.e.)* |
|---|---|---|---|
| 2 | 5 | 3.8 | 20.1 ± 1.5 |
| 3 | 4 | 2.6 | 37.6 ± 3.2 |
| 4 | 5 | 2.5 | 50.5 ± 4.0 |
| 5 | 5 | 2.3 | 54.0 ± 3.5 |
| 6 | 4 | 1.8 | 64.0 ± 6.8 |
| 7 | 5 | 3.2 | 56.9 ± 3.2 |
| 8 | 4 | 2.6 | 50.8 ± 5.9 |
| 9 | 5 | 2.9 | 56.0 ± 3.1 |
| 10 | 4 | 5.9 | 42.6 ± 7.4 |
| 11 | 5 | 5.7 | 33.4 ± 3.9 |
| 12 | 5 | 4.7 | 16.0 ± 3.4 |
| 13 | 4 | 5.2 | 8.6 ± 2.4 |

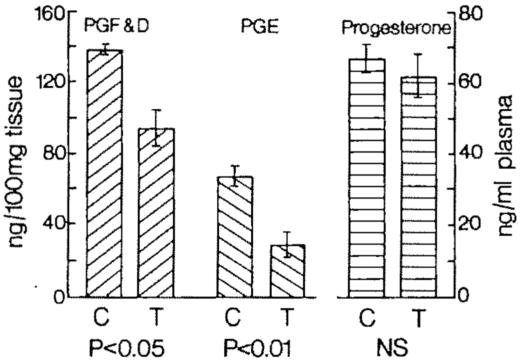

**Figure 2** Effect of tamoxifen on production of prostaglandin F and D (PGF & D) and prostaglandin E (PGE) by rat uterus homogenates on day 5 of pseudopregnancy. Peripheral plasma levels of progesterone are also shown. C=control (4 rats); T=tamoxifen-treated (5 rats).

5 ($P<0.01$). From this peak, prostaglandin E levels gradually fell to reach low levels between days 10 and 13.

A comparison of the ratios of the levels of prostaglandins F and D to the level of prostaglandin E is interesting. This ratio steadily decreases from days 2 to 6, possibly indicating that prostaglandin E production is stimulated more than that of F and D. The ratio then shows a sudden rise on day 7, since prostaglandin F and D levels increase on that day while prostaglandin E levels fall. The ratio falls slightly on days 8 and 9 before rising to its highest values between days 10 and 13. This high ratio is due to the very low level of prostaglandin E production rather than to a high level of production of F and D.

Plasma progesterone levels (Table 1) increase rapidly during the first 3 days of pseudopregnancy reaching high levels by day 4 or 5. The levels then show small fluctuations up to day 9, after which they decline to much lower levels by day 13. It may be that the increase in production of prostaglandins F and D from day 8 to 9, and the relatively higher production of prostaglandins F and D in comparison to E from days 10 to 13 may be connected with this decline. It is clear that the much higher production of prostaglandins F and D on day 5 is not connected with any decline in progesterone levels.

### *Effect of tamoxifen on prostaglandin production by the pseudopregnant rat uterus* in vitro

The results are shown in Figure 2. The pretreatment of rats on day 2 with tamoxifen significantly decreased the *in vitro* production of prostaglandins by the day 5 pseudopregnant rat uterus. However, the production of prostaglandin E was apparently affected more since the ratio of prostaglandins F and D to E was 2.0 in the control animals, but increased to 3.5 in the drug-treated animals. Peripheral plasma progesterone levels were high in both groups, as expected, and did not differ.

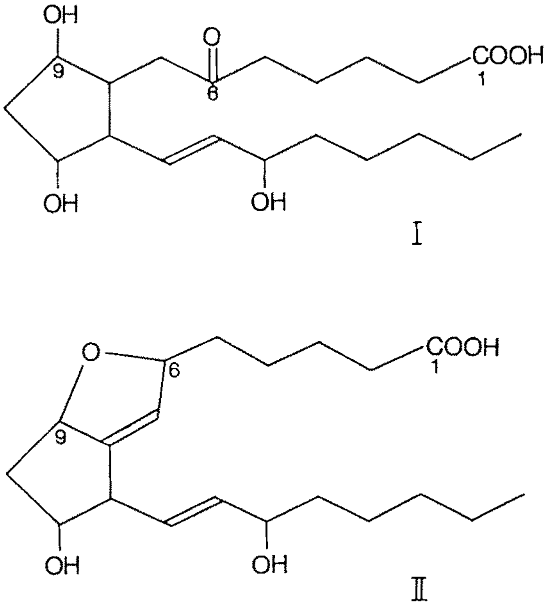

**Figure 3** Structures of 6-keto prostaglandin $F_1\alpha$ (I) and 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid (II).

### *Identification of prostaglandins*

*'F' fraction.* The predominant prostaglandin present was $F_{2\alpha}$ in all samples studied. Much smaller quantities (1 to 5%) of $F_{1\alpha}$ were also detectable.

*'E' fraction.* Prostaglandin $E_2$ was identified as being present in all samples. Smaller quantities of prostaglandin $D_2$ were also present in each sample. Neither prostaglandin $E_1$ nor 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ were detected. The major product present in each sample was 6-keto-prostaglandin $F_{1\alpha}$ (Figure 3). The identification of this compound by GC-MS in the remaining uterine extracts forms an addendum to this paper. It was estimated to be present in quantities at least 10 to 20 times more than the quantities of prostaglandin $E_2$, and, therefore, was also present in greater quantities than $F_{2\alpha}$. It was impossible to measure the quantities accurately since the authentic compound was not available.

Also present was another prostaglandin derivative. Its mass spectrum was identical to that of a compound produced by rat stomach homogenates and which has been identified as 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid (Pace-Asciak and Wolfe, 1971a) (Figure 3). It was present in quantities less than 6-keto-prostaglandin $F_{1\alpha}$ but probably more than $F_{2\alpha}$. An accurate measurement of the amounts again was not possible.

## Discussion

It was unexpected that a major prostaglandin produced by rat uterus homogenates would be 6-keto-prostaglandin $F_{1\alpha}$. The isolation of this prostaglandin from the uterus has not been previously reported. 6-Keto-prostaglandin $F_{1\alpha}$ was produced in amounts at least 5 to 10 times greater than the amounts of $F_{2\alpha}$ the prostaglandin in next abundance. We did not have any authentic 6-keto-prostaglandin $F_{1\alpha}$ to test its cross-reactivity with the $F_{2\alpha}$ antibody. Prostaglandin $F_{1\alpha}$ cross-reacts 100%, but the introduction of a 6-keto group may reduce the cross-reactivity. Perhaps of more significance was the finding that 6-keto-prostaglandin $F_{1\alpha}$ is similar in polarity to prostaglandin $E_2$. It was eluted in the 'E' fraction from the silicic acid columns and its retention time of gas chromatography was 1 to 2 min longer than that of prostaglandin $E_2$. This is despite that fact that 6-keto-prostaglandin $F_{1\alpha}$ contains 3 hydroxyl groups while $E_2$ contains only 2 such groups. This probably indicates that the 6-keto group and 9-hydroxyl group interact in some way, resulting in a reduction in polarity of the molecule. This interaction may also reduce the affinity of 6-keto-prostaglandin $F_{1\alpha}$ for the $F_{2\alpha}$ antibody. It is difficult to predict what the cross-reactivity would be, and, therefore, what contribution 6-keto-prostaglandin $F_{1\alpha}$ makes to the final quantitative answer in the assay of prostaglandin F compounds.

It was also unexpected that 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid would be produced by rat uterus homogenates, though it is known to be produced by the rat stomach (Pace-Asciak & Wolfe, 1971a). This compound probably has low cross-reactivity with the prostaglandin $F_{2\alpha}$ antibody since in comparison to $F_{2\alpha}$ its structure has been modified at both the 6 and 9 positions.

Prostaglandin $D_2$ was also detected in the uterine incubates. This cross-reacts 17% with the prostaglandin $F_{2\alpha}$ antibody, but since much less of it was produced, it probably contributes only 3 to 5% to the final quantitative value. Prostaglandin $F_{1\alpha}$ cross-reacts 100%, but it was present in the incubates in trace amounts. It will contribute only 1 to 3% to the final answer. Consequently, in the assay using the prostaglandin $F_{2\alpha}$ antibody, prostaglandin $F_{2\alpha}$ is a major component being assayed and possibly 6-keto-prostaglandin $F_{1\alpha}$ is another. However, due to the lack of adequate specificity, one is actually measuring the levels of prostaglandin F and D compounds. This does not include the metabolites of prostaglandin $F_{2\alpha}$ (e.g. 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$) since these do not have significant cross-reactivities.

The antiserum against prostaglandin $E_2$was probably measuring exclusively prostaglandin $E_2$, unless 6-keto-prostaglandin $F_{1\alpha}$ and 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid unexpectedly have high cross-reactivities. Prostaglandin $E_1$ is the only prostaglandin which cross-reacts significantly with the $E_2$ antibody, and its presence was not detected.

From the quantitative results, it is seen that prostaglandin production is highest on day 5 of pseudopregnancy. The level measured is the sum of the amount already present in the uterus plus that synthesized during incubation minus the amount metabolized. 13,14-Dihydro-15-keto-prostaglandin (which is similar in polarity to prostaglandin $E_2$) was not detected. It would appear that in this *in vitro* system, metabolism of prostaglandins is low and we were actually measuring prostaglandin synthesis by the uterus. However, a detailed study of metabolism is merited. Day 5 is the day of implantation in the rat and it is possible, that a high production of prostaglandins on day 5 is connected with this process.

The increase in production of prostaglandins by the uterus on day 5 of pseudopregnancy was significantly inhibited by treating the rats with tamoxifen on day 2. Tamoxifen, at this dose level, inhibits the output of oestradiol from the ovary (Watson *et al.*, 1975). This would indicate that the increase in prostaglandin production normally occurring from day 3 to day 5 is linked to the increased output of oestradiol from the ovary which occurs late on day 3 and early on day 4. These results also suggest that drugs which inhibit the release and/or action of hormones may be of importance in inhibiting prostaglandin synthesis. In this study, other mechanisms of action of tamoxifen cannot be ruled out.

Ham *et al.* (1975) showed that the treatment of ovariectomized rats with oestradiol stimulated prostaglandin $F_{2\alpha}$ synthesis by the uterus but reduced $E_2$ synthesis. This resulted in an increase in the ratio of prostaglandins F to E. This did not occur in the day 5 uterus in the present study. Synthesis of all prostaglandins was apparently stimulated with prostaglandin $E_2$ synthesis actually being stimulated more, as the ratio of prostaglandins F and D to E decreased. It is difficult to explain this difference in findings except that in this study progesterone levels were high, while in the study of Ham *et al.* (1975), the ovariectomized rats were not receiving progesterone supplements.

It was also surprising that prostaglandin production did not increase greatly towards the end of pseudopregnancy when oestradiol levels are high. The ratio of prostaglandin F and D to E did increase, and possibly this is of significance. Uterine levels of prostaglandin $F_{2\alpha}$ do not increase greatly towards the end of pseudopregnancy, though they are significantly higher around days 9 and 10 (Weems *et al.*, 1976; Castracane & Shaikh, 1976). Oestrogen does cause the release of prostaglandin F from the uterus of ovariectomized rats though a period of progesterone priming is necessary for the maximum effect to be produced (Castracane & Jordan, 1975). Whether or not prostaglandin $F_{2\alpha}$ produced by the rat uterus is the factor which terminates pseudopregnancy has still to be proven. Our finding that 6-keto-prostaglandin $F_{1\alpha}$ is the major product formed by rat uterus homogenates, raises the possibility that this compound may be the uterine luteolytic hormone in the rat. Consequently, many of the earlier studies may need to be re-appraised in the light of our present findings.

This study was supported by the M.R.C. Tamoxifen was kindly donated by Dr A. Walpole, ICI Ltd., Alderley Park, Macclesfield, Cheshire and prostaglandins by the Upjohn Co., Michigan, U.S.A. The technical assistance of Mrs L. Simon, who performed the prostaglandin E assays, is greatly appreciated.

## Addendum

# IDENTIFICATION OF 6-KETO-PROSTAGLANDIN $F_{1\alpha}$ BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

## Introduction

The identification of a compound by gas chromatography and mass spectrometry involves the formation of different chemical derivatives and the interpretation of the mass spectra produced following gas chromatography. It is essential to choose derivatives which identify the nature and position of the functional groups present. Since the extraction and chromatographic procedures described in the preceding paper are directed towards isolating prostaglandins, it was highly probable that the new compound isolated had a prostaglandin-like structure. The determination of the structure of this compound is the subject of this addendum.

## Methods

Ethyl acetate extracts of pseudopregnant rat uteri were pooled to produce several larger samples consisting of 4 or 5 individual samples. Each pooled sample was evaporated to dryness, the residue dissolved in 67% ethanol and washed with petroleum ether (b.p. 60° to 80°C). The ethanol solution was evaporated to dryness and the residue further purified by column chromatography, as described in the preceding paper.

The 'E' fraction from each column was subjected to derivatization for analysis by gas chromatography-mass spectrometry. Methyl ester formation, *n*-butyloximation of the keto groups, and trimethylsilyl ether formation were performed as described in the preceding paper. Ethyl esters were prepared using diazoethane, and methyloximes formed with O-methylhydroxylamine hydrochloride (5 mg/ml in dry pyridine). Cyclic *n*-butylboronates were formed by reacting the esterified compound with *n*-butylboronic acid (5 mg/ml in 2,2-dimethoxypropane) for 60 min at 60°C.

The keto group in the unknown compound was reduced also with sodium borohydride in ethanol at room temperature for 30 minutes. After the addition of water and acidification to pH 4.5 with 1 N HCl, the reduced compound was extracted with ethyl acetate. It was then converted into the methyl or ethyl ester, trimethylsilyl ether. Seven derivatives of the unknown compound were prepared.

### *Analytical procedure*

The derivatives were analysed on an LKB 9000 gas chromatograph-mass spectrometer. The glass column, 3 m × 3 mm, was packed with 3% OV1 on Supelcoport. The helium gas flow was 35 ml/minute. The column temperature was 245°C, or 248°C when oximes were analysed. A mixture of straight chain fatty acid methyl esters (consisting of 16 to 24 carbons) was run before each sample in order to calculate its carbon value (C value).

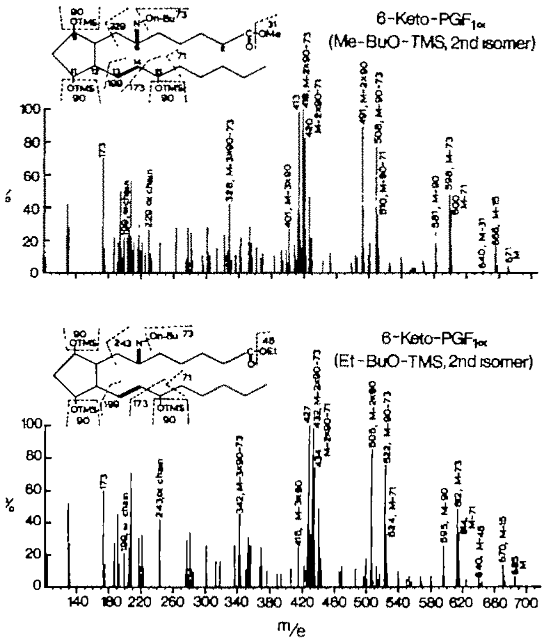

Figure 4 Line diagrams of the mass spectra of the methyl ester, *n*-butyloxime, trimethylsilyl ether (Me-BuO-TMS) and the ethyl ester, *n*-butyloxime, trimethylsilyl ether (Et-BuO-TMS) of the compound isolated and identified as 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PG$F_{1\alpha}$). Ordinate scale: relative abundance of ions (as % of base peak); abscissa scale: ratio of mass (m) to charge (e) (where e = 1).

## Results and Discussion

(1) *Methyl ester,* O-n-*butyloxime, trimethylsilyl ether (Me-BuO-TMS)*

Two oxime isomers were obtained which separated on gas chromatography (C values = 26.5 and 26.8). The mass spectrum of the second isomer is shown in Figure 4. The following points are noted:

(a) The molecular ion (mol. ion) occurs at m/e 671 (M) and prominent peaks are found at m/e 598 (M-73), 581 (M-90), 508 (M-90-73), 491 (M-2 × 90), 418 (M-2 × 90 -73), 401 (M-3 × 90) and 328 (M-3 × 90 - 73). These data establish the presence of 3 hydroxyl groups and one keto group and are consistent with a mono-keto prostaglandin $F_{1\alpha}$ structure.

(b) Peaks at m/e 600 (M-71), 510 (M-90-71) and 420 (M-2 × 90 -71) and at 173 and 199 strongly suggest that the ω-chain has the primary prostaglandin structure (i.e. a 13,14 double bond and a 15-hydroxyl group). Also the keto group cannot be situated in the ω-chain.

(c) The peak at m/e 229 indicates that the keto group is probable situated in the α-chain.

(2) *Ethyl ester,* O-n-*butyloxime, trimethylsilyl ether (Et-BuO-TMS)*
Two isomers were detected on gas chromatography with C values of 26.8 and 27.0 respectively. The mass spectrum of the second isomer is shown in Figure 4. This is similar to the Me-BuO-TMS derivative except that the major peaks, with two exceptions, are 14 mass units higher. The most significant finding is a peak at m/e 243 instead of m/e 229. This indicates that the keto group is in the α-chain, and not in the ω-chain which still produces a peak at m/e 199.

(3) *Methyl ester, methyloxime, trimethylsilyl ether (Me-Mo-TMS)*
Two isomers were produced which were resolved by gas chromatography. The first isomer (C value = 24.8) was much smaller than the second isomer (C value = 25.1). A mass spectrum taken of the second isomer showed the mol. wt. of this derivative to be 629. This is due entirely to a difference of 42 mass units between the n-butyloxime and the methyloxime. This clearly indicates that there is only one keto group in the molecule. Furthermore, a peak at m/e 187 (229-42) again showed that the keto group is in the α-chain.

(4) *Methyl ester, cyclic* n-*butyl boronate, trimethylsilyl ether (Me-BuB-TMS)*
The production of butylboronates requires two hydroxyl groups to be present in a 1,3 *cis*-configuration, with the formation of a bridge across these two hydroxyl groups to produce a cyclic derivative. This can only occur in the $F_\alpha$ prostaglandins where hydroxyl groups are situated in the 9α and 11α positions (Pace-Asciak & Wolfe, 1971b; Kelly, 1972). (Both hydroxyl groups could be in the β positions, but such prostaglandins do not occur naturally). Consequently, the very fact that the compound under study formed a cyclic *n*-butylboronate indicates that this compound contains the prostaglandin $F_\alpha$ structure. The mol. ion of the Me-BuB-TMS derivative (C value = 25.2) was 522. This is consistent with the compound having the prostaglandin $F_{1\alpha}$ structure with a keto group in the α-chain. Only two major peaks occurred in its mass spectrum. The smaller peak was at m/e 365, which is equivalent to M–α-chain (522–157). The base peak is at m/e 129 and is probably due to the ion ($CH_2{=}CH{-}CH{=}\overset{+}{O}{-}TMS$).

(5) *Ethyl ester, cyclic* n-*butyl boronate, trimethylsilyl ether (Et-BuB-TMS)*
The carbon value of this derivative was 25.5 and its mol. wt. was 536. Two major peaks occurred in its mass spectrum, again at m/e 365 (M–α-chain, where the α-chain is now 171) and at m/e 129 indicating

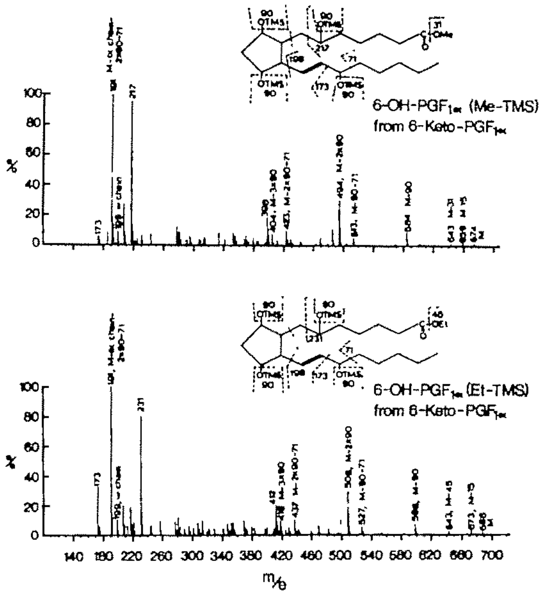

Figure 5 Line diagrams of the mass spectra of the methyl ester, trimethylsilyl ether (Me-TMS) and the ethyl ester, trimethylsilyl ether (Et-TMS) of 6-hydroxyl-prostaglandin $F_{1\alpha}$ (6-OH-PGF$_{1\alpha}$) prepared by the reduction of the isolated compound identified as 6-keto-prostaglandin $F_{1\alpha}$. Ordinate scale: relative abundance of ions (as % of base peak); abscissa scale: ratio of mass (m) to charge (e) (where e=1).

identical fragmentation as in the Me-BuB-TMS derivative.

The evidence accumulated from these 5 derivatives indicates that the compound isolated consists of a prostaglandin $F_{1\alpha}$ structure with a keto group attached to the α-chain. The fragmentation patterns of the derivatives prepared so far give little indication where the keto group is actually located on the α-chain. It was decided, therefore, to reduce the keto group to a hydroxyl group since carbon-carbon bonds split α to a carbon-oxygen bond (as in the loss of 71 and 173 around the carbon-oxygen bond at position 15; Figure 4). The fragmentation pattern of the α-chain should, establish the position of the keto groups.

(6) *Methyl ester, trimethylsilyl ether (Me-TMS) of the reduced compound*
The reduction of the keto group produces two isomers. Our experience of 15(R) and 15(S) prostaglandin $F_{2\alpha}$ has shown that these two isomers do not separate on gas chromatography. It was no surprise, therefore, that the reduced form of the compound under study produced only one gas chromatographic peak (C value=25.0). Its mass spectrum is shown in Figure 5 and the following points are noted: (i) The mol. wt. is 674, which is consistent with the compound being prostaglandin $F_{1\alpha}$ containing one extra hydroxyl group. (ii) A major peak occurs at m/e 217. As shown in the figure, this most probably arises from a split of the bond between carbons 6 and 7, which is α to the carbon-oxygen bond. (iii) The base peak at m/e 191 is due to the loss of the α chain, the two derivatized hydroxyl groups from the cyclopentane ring and the loss of the 5 terminal carbons from the ω-chain. (iv) Since peak m/e 191 is formed preferentially, peaks at m/e 173 and 199 are correspondingly small. (v) There are several significant peaks indicating the loss of derivatized hydroxyl groups (90) and the 5 terminal carbons (71). The peak at m/e 584 (M-90) corresponds to the mol. wt. of prostaglandin $F_{2\alpha}$ (Me-TMS) and the mass spectrum between m/e 300 and 600 is remarkably similar to that of prostaglandin $F_{2\alpha}$ (not $F_{1\alpha}$ since one would have to add 2 protons to compensate for the loss of one derivatized hydroxyl group) (see Poyser, 1972, for a mass spectrum of prostaglandin $F_{2\alpha}$-Me-TMS).

(7) *Ethyl ester, trimethylsilyl ether (Et-TMS) of the reduced compound*
Only one peak was produced on gas chromatography (C value=25.2). The mass spectrum of the Et-TMS of the reduced compound is shown in Figure 5: (i) The mol. wt. is 688, 14 mass units higher than the Me-TMS derivative, as expected. (ii) The base peak is at m/e 191, as expected also, since the production of this fragment involves loss of the α-chain and is, therefore, not affected by the ester derivative. (iii) If the derivatized hydroxyl group is located at position 6, one would now expect the m/e 217 peak present in the Me-TMS derivative to move to m/e 231 in the Et-TMS derivative. A comparison of the mass spectra shows this to be the case, proving conclusively that the hydroxyl group (and, therefore, the original keto group) is located on the carbon at position 6.

## Conclusion

The evidence obtained from this study clearly indicates that the new compound isolated from the pseudopregnant rat uterus is 6-keto-prostaglandin $F_{1\alpha}$. This conclusion does make the assumption that both hydroxyl groups on the cyclopentane ring are in the α positions, and that the double bond in the ω-chain is in the 13,14 position. However, naturally-occurring prostaglandins of the F series do have this configuration so hopefully these assumptions are justified. Absolute identification must await the chemical synthesis of the authentic compound and a comparison being made of the mass spectra of the naturally-occurring and synthetic material.

Finally, whilst this study was in progress a short report was published showing that 6-keto-prostaglandin $F_{1\alpha}$ is also formed by homogenates of rat stomach (Pace-Asciak, 1976).

We appreciate the help of Dr C.J. Thompson in the computerized drawing of the line diagrams. This study was supported by an M.R.C. grant to Professor E.W. Horton.

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*(Received July 1, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 201–208

# ANAPHYLACTIC CONTRACTION OF PULMONARY BLOOD VESSELS OF CHICKEN

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1 Isolated pulmonary arterial and vein strips from sensitized or non-sensitized chickens exhibited dose-dependent contractions to adrenaline >, noradrenaline >, 5-hydroxytryptamine >, histamine >, dopamine. Individual variability in the responsiveness of the vessels to agonists was marked. In general veins were 2 to 25 times more sensitive to agonists than arterial strips.

2 Isoprenaline (a relatively specific $\beta$-adrenoceptor agonist) induced relaxations of the submaximally contracted pulmonary vein and arteries at low doses and contractions at high concentrations.

3 Contractile responses to acetylcholine or carbachol were not regularly recorded; only 50% of the vessels reacted to cholinoceptor agonists over a wide threshold dose range.

4 Chicken pulmonary vessels were found relatively insensitive to bradykinin.

5 Effects of prostaglandins were variable. Prostaglandin $F_{2\alpha}$ induced dose-related contractions of the vein and arterial strips; prostaglandins $E_1$ and $E_2$ at low doses relaxed partially contracted pulmonary artery irrespective of the spasmogen used and further increase in doses induced either no effect or contractions. Prostaglandin $E_1$ induced marked and rapid contractions of the vein. Prostaglandin $E_2$ induced relaxations of the prostaglandin $F_{2\alpha}$-contracted vein only, but produced no effect or slight contractions of the veins partially contracted to other spasmogens.

6 Pulmonary arterial and vein strips obtained from chickens sensitized to horse plasma exhibited Schultz-Dale contractions of variable magnitude and duration to specific antigenic challenge only. In many vessels, antigen-induced contractions were associated with marked increase in spontaneous activity.

7 The importance of the Schultz-Dale reaction in avian pulmonary vessels is discussed in relation to the right heart dilatation associated with anaphylaxis in the chicken.

## Introduction

Specific antigen-induced contraction of smooth muscle taken from sensitized tissue is known as a Schultz-Dale phenomenon (Schultz, 1910; Dale, 1913). This immunopharmacological reaction has subsequently been demonstrated in numerous tissues: the guinea-pig ileum (Dale & Okpako, 1969; Dale & Zilleti, 1970; Cîrstea, 1970; Okpako, 1970) and trachea (Sörenby, 1975); rat uterus (Kellaway, 1930); human tracheal and bronchial strips (Schild, Hawkins, Mongar & Herxheimer, 1951; Schild, 1956); pulmonary artery of the rabbit (Lecomte, 1958) and calf (Eyre, 1970; 1975); calf pulmonary veins (Eyre, 1970; 1971a; 1973; 1975; Eyre & Deline, 1971a, b; 1972), hepatic veins (Holroyde & Eyre, 1975) and digital veins (Elmes & Eyre, unpublished); pulmonary vein of the horse (Eyre, 1972; Burka, Deline, Holroyde & Eyre, 1976) and recently several gastrointestinal tissues of the domestic fowl (Chand & Eyre, 1976). Surprisingly, sheep pulmonary vein has been shown to relax to antigen (Eyre, 1975).

Sensitized adult domestic fowl exhibit antigen-dose-dependent increases in the central venous pressure associated with systemic arterial hypotension (Chand & Eyre, unpublished). Birds that die in anaphylaxis show right heart dilatation (Lecomte & Beaumariage, 1958; Aronson, Bilstad & Wolfe, 1961; Chand & Eyre, unpublished) which has also been described as a common feature of systemic anaphylaxis in man, mouse rabbit and chicken; attributable to increased pulmonary vascular resistance (Aronson *et al.*, 1961).

Thus, it was important to investigate the possibility of demonstrating the Schulz-Dale reaction in pulmonary blood vessels of chickens and to study the effects of catecholamines, 5-hydroxytryptamine, acetylcholine, histamine, bradykinin and prostaglandins at the same time.

## Methods

Adult domestic fowl (White Leghorn) weighing 2 to 4 kg, were sensitized to horse plasma (1 ml/kg, i.v.)

(Chand & Eyre, 1976). On the 7th day after sensitization, chickens were killed with pentobarbitone sodium. Immediately, combined heart and lungs were carefully removed and kept in aerated ice cold Krebs solution. Probes of appropriate diameter were passed into the pulmonary artery and vein from their points of cardiac origin. The vessels were carefully detached from surrounding lung tissues and then cut helically following the method of Furchgott & Bhadrakom (1953) to give a strip of about 2 cm length and 2 to 3 mm width containing predominantly circular smooth muscle fibres. Arterial and venous strips from individual chickens were suspended in 30 or 50 ml organ baths containing Krebs-Henseleit solution maintained at 37°C, bubbled with 5% $CO_2$ in $O_2$. Tissues were allowed to equilibrate for 1 h under a resting tension of 0.5 g for pulmonary vein and 1 g for pulmonary artery. Pulmonary vessel strips from unsensitized chickens were set up in the same way.

Three or four reproducible responses (single or cumulative) of each strip to 3 or 4 agonists at 15 to 30 min intervals were recorded with an E & M isotonic myograph transducer connected to an E & M Desk Model, 4-Channel Physiograph (DMP-4B) pen recorder (Narco Instruments, Houston, Tex.). The strips were then 'challenged' with 0.2 to 0.3 ml of horse plasma. In some experiments, after first challenge, strips were washed 2–3 times and challenged within 15–30 min with a second identical dose of antigen to study the extent of desensitization. Subsequently, the strips were washed and allowed to rest for 1 to 3 hours. Responsiveness to one or more agonist(s) was checked and the tissues were again challenged with antigen to study the extent of recovery of the Schultz-Dale response.

### *Drugs*

The following drugs were used: histamine diphosphate, serotonin creatinine sulphate (5-hydroxytryptamine, 5-HT), bradykinin triacetate, carbamylcholine chloride (carbachol), acetylcholine chloride (ACh), adrenaline bitartrate, noradrenaline bitartrate, dopamine hydrochloride, (Nutritional Biochemical Corp., Cleveland, Ohio), isoprenaline hydrochloride, (Wintrobe, New York) and prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ (gifts from Upjohn Co., Kalamazoo, Mich.).

## Results

### *Vascular reactivity to drugs*

Pulmonary artery and pulmonary vein of the fowl exhibited dose-dependent contractions to adrenaline, noradrenaline, 5-HT, dopamine, histamine, ACh, carbachol and prostaglandin $F_{2\alpha}$. Vein strips were 2 to 25 times more reactive than artery strips to most of the agonists tested. Threshold dose-ranges to agonists

**Table 1** The threshold dose-ranges of some vasoactive substances on isolated spiral strips of pulmonary artery and vein of adult domestic fowl

| | *Threshold dose-ranges (M)* | |
|---|---|---|
| *Vasoactive substances* | *Pulmonary artery* | *Pulmonary vein* |
| Histamine | $10^{-5}$ to $5 \times 10^{-4}$ | $10^{-6}$ to $10^{-4}$ |
| 5-Hydroxytryptamine | $5 \times 10^{-8}$ to $10^{-5}$ | $10^{-8}$ to $10^{-5}$ |
| Acetylcholine | $5 \times 10^{-6}$ to $10^{-4}$ | $10^{-5}$ to $5 \times 10^{-4}$ |
| Carbachol | $10^{-7}$ to $10^{-4}$ | $10^{-6}$ to $10^{-4}$ |
| Adrenaline | $2 \times 10^{-8}$ to $5 \times 10^{-7}$ | $10^{-9}$ to $2 \times 10^{-7}$ |
| Noradrenaline | $5 \times 10^{-8}$ to $5 \times 10^{-7}$ | $5 \times 10^{-9}$ to $2 \times 10^{-7}$ |
| Dopamine | $2 \times 10^{-6}$ to $10^{-5}$ | $10^{-6}$ to $5 \times 10^{-6}$ |
| Isoprenaline | $10^{-8}$ to $10^{-7}$ R | $10^{-8}$ to $10^{-7}$ R |
| | $10^{-6}$ to $10^{-5}$ C | $10^{-6}$ to $10^{-5}$ C |
| Bradykinin µg/ml | 10 to 50 | 10 to 50 |
| $PGE_1$ ng/ml | 10 to 500 R | 50 to 500 C |
| µg/ml | 2 to 5 C | |
| $PGE_2$ ng/ml | 10 to 100 R | 10 to 100 $R^1$ |
| µg/ml | 2 to 5 C | 1 to 5 C |
| $PGF_{2\alpha}$ µg/ml | 0.1 to 1 C | 0.1 to 0.5 |
| * Horse plasma (ml/50 ml) | 0.01 to 0.1 | 0.01 to 0.1 |

* Only on sensitized tissues; R = Relaxation of blood vessel strips partially contracted to histamine, 5-HT, catecholamines, prostaglandins or antigen. $R^1$ = Relaxation of only prostaglandin $F_{2\alpha}$-contracted vein; C = Contraction.

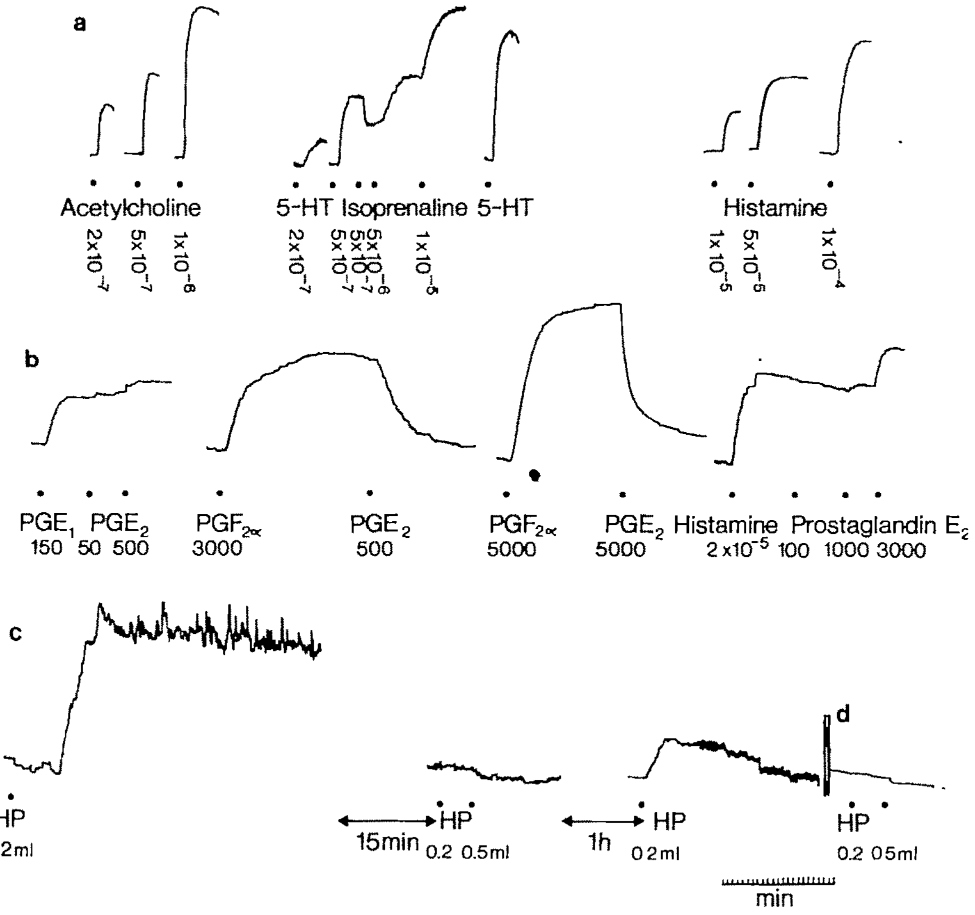

**Figure 1** Three (a, b and c) isolated spiral stips of pulmonary veins of sensitized adult domestic fowl. Strip (a) shows typical dose-related contractile responses to acetylcholine, 5-hydroxytryptamine (5-HT) and histamine; also relaxant effect of low dose of isoprenaline on partially 5-HT-contracted vein, and contraction to higher doses of isoprenaline. Strip (b) shows contractile responses to prostaglandins $E_1$, and $F_{2\alpha}$ ($PGE_1$, $PGF_{2\alpha}$) and histamine. $PGE_1$-contracted vein is further contracted by prostaglandin $E_2$ ($PGE_2$). $PGF_{2\alpha}$-contracted vein is relaxed by $PGE_2$ in dose-dependent fashion. Histamine-contracted vein is further contracted by high doses of $PGE_2$. Strip (c) shows a Schultz–Dale anaphylactic reaction to specific sensitizing antigen (horse plasma). Subsequent challenge to the same antigen after 15 min exhibits desensitization. Allowing the strip to 'rest' for 1 h with frequent washing results in partial recovery of anaphylactic response. Additional strips (d) obtained from nonsensitized chickens, exhibited similar responses to agonists as sensitized strips, but did not contract to even higher doses of horse plasma. Time marker indicates minutes. Concentrations of agonists are molar bath concentrations except prostaglandins which are ng/ml. Horse plasma (HP) ml per 50 ml bath.

presented in Table 1, clearly show considerable variability in reactivity to the drugs tested. Characteristic responses to ACh, histamine, 5-HT and prostaglandins are shown in Figures 1 and 2 (a and b). Many vein (7/15) and arterial (5/17) strips did not react to ACh or carbachol ($10^{-5}$ to $10^{-3}$ M), while others exhibited dose-dependent ($10^{-7}$ to $10^{-5}$ M) rapid contractions.

Histamine was found to be 100 to 1000 times less active than 5-HT, noradrenaline or adrenaline. Some (4/15) pulmonary arterial strips were 2 to 15 times more responsive to histamine than the corresponding veins. A few of the veins (4/18) and arterial (5/15) strips exhibited extremely low reactivity to histamine (threshold $> 10^{-4}$ M).

Although vein strips were generally more responsive than arteries to 5-HT, in some cases (5/23) the arteries were the more responsive. 5-HT,

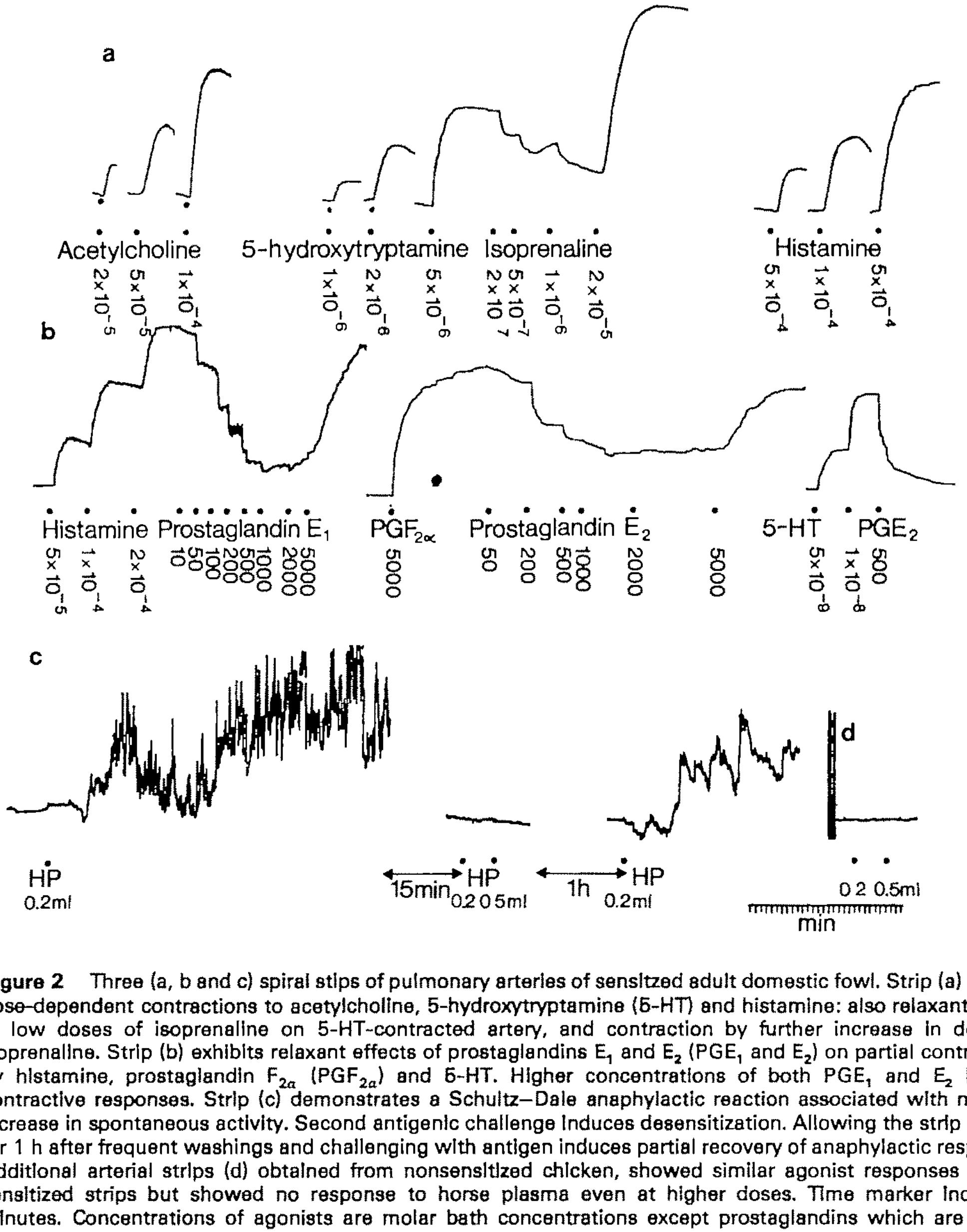

**Figure 2** Three (a, b and c) spiral stips of pulmonary arteries of sensitzed adult domestic fowl. Strip (a) shows dose-dependent contractions to acetylcholine, 5-hydroxytryptamine (5-HT) and histamine: also relaxant effect of low doses of isoprenaline on 5-HT-contracted artery, and contraction by further increase in dose of isoprenaline. Strip (b) exhibits relaxant effects of prostaglandins $E_1$ and $E_2$ ($PGE_1$ and $E_2$) on partial contraction by histamine, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 5-HT. Higher concentrations of both $PGE_1$ and $E_2$ induce contractive responses. Strip (c) demonstrates a Schultz–Dale anaphylactic reaction associated with marked increase in spontaneous activity. Second antigenic challenge induces desensitization. Allowing the strip to rest for 1 h after frequent washings and challenging with antigen induces partial recovery of anaphylactic response. Additional arterial strips (d) obtained from nonsensitized chicken, showed similar agonist responses to the sensitized strips but showed no response to horse plasma even at higher doses. Time marker indicates minutes. Concentrations of agonists are molar bath concentrations except prostaglandins which are ng/ml Horse plasma (HP), ml per 50 ml bath.

adrenaline and noradrenaline were approximately equieffective. Dopamine however was 100 to 1000-fold less potent than the other catecholamines.

Isoprenaline ($10^{-8}$ to $5 \times 10^{-6}$ M) produced dose-related relaxations of pulmonary veins and arteries that were partially contracted to histamine, 5-HT, prostaglandin $F_{2\alpha}$ or horse plasma. Further increase in the isoprenaline concentration ($10^{-5}$ to $5 \times 10^{-5}$ M) induced dose-dependent contractions. Arterial preparations were consistently more sensitive than veins to isoprenaline (Figures 1a & 2a).

Bradykinin (1 to 50 µg/ml) tested on a limited number of veins ($n=4$) and arteries ($n=5$) exhibited weak contractile responses subject to tachyphylaxis.

Prostaglandin $F_{2\alpha}$ (0.1 to 10 µg/ml) produced dose-dependent contractions of both artery and vein strips.

Effects of prostaglandins $E_1$ and $E_2$ were variable. Arterial strips that were contracted submaximally to histamine, 5-HT, prostaglandin $F_{2\alpha}$, carbachol or antigen were relaxed by prostaglandins $E_1$ and $E_2$ (10 to 1000 ng/ml) in dose related fashion; further increase in the concentrations (1 to 10 µg/ml) produced either no effect or contractile responses of variable magnitude (Figure 2b).

Prostaglandin $E_1$ (50 ng to 5 µg/ml) always produced dose-dependent contractions of the pulmonary vein. Prostaglandin $E_2$ (100 ng to 5 µg/ml) caused dose-related relaxations in prostaglandin $F_{2\alpha}$-contracted vein strips only and showed no effect or slight contractile responses of veins contracted by prostaglandin $E_1$. In contrast, prostaglandin $E_2$ produced further contraction of veins that were partially contracted to histamine and 5-HT (Figure 1b).

### *Schultz–Dale response*

The incidence of Schultz–Dale reactions in pulmonary blood vessels of chicken is given in Table 2. Vessels obtained from non-sensitized chickens exhibited responses to agonists similar to those of sensitized birds, but none of the control veins ($n=6$) or arteries ($n=7$) contracted to horse plasma (0.2 to 0.5 ml in 30 to 50 ml bath). Sixty-five % of pulmonary arteries and 76% of the veins obtained from chickens sensitized to horse plasma contracted to 0.2 ml horse plasma but did not react to non-specific antigens (bovine plasma, duck plasma, crystalline albumins of dog, cow, rabbit) over wide dose-ranges. The character of the Schultz–Dale response was variable with respect to the latent period (30 s to 5 min), duration (5 to 30 min), magnitude (10–90% of the maximum) and degree of spontaneity. Increased spontaneous activity was seen in 50% of the vessels. No correlation could be found between vascular reactivity to agonists and antigen. Some strips weakly sensitive to histamine, 5-HT or carbachol ($10^{-5}$ to $10^{-4}$M) reacted strongly to specific antigen. In a few, the reverse of this was found. The second antigen 'challenge' with the same or higher dose 15 to 30 min after the first challenge, produced either no response or markedly reduced reaction. After allowing the strips to 'rest' for 1 to 3 h there was no change in reactivity to agonists, but partial recovery in responsiveness to the antigen (Figures 1 and 2, c) was recorded.

## Discussion

In this study most of the pulmonary vein and artery strips of the chickens contracted dose-dependently to catecholamines, histamine, 5-HT, ACh and carbachol. Earlier Somlyo, Somylo & Woo (1967) reported that pulmonary arterial strips of adult fowl are insensitive to ACh, histamine, 5-HT and bradykinin. The reasons for this discrepancy are difficult to assess but may be associated with differences in age and breed. The variations and irregular responses of avian pulmonary vessels to ACh and carbachol were not surprising, since similar findings have been noted earlier with bovine pulmonary (Burka & Eyre, 1974), mesenteric, hepatic (Holroyde & Eyre, 1975), and digital vessels (Elmes & Eyre, unpublished).

Isoprenaline produced dose-dependent relaxations of both pulmonary artery and vein at low doses and contractions at higher doses. Similar responses to isoprenaline have earlier been shown in the avian anterior and posterior mesenteric and pancreatico-duodenal veins (Bolton & Bowman, 1969), rabbit aorta (Furchgott & Bhadrakom, 1953), calf mesenteric vein (Holroyde & Eyre, 1975) and pulmonary artery and vein (Eyre, 1971b). In some of the blood vessels a complete lack of $\beta$-adrenoceptor (inhibitory adrenergic) mechanisms has been reported (Somlyo & Somlyo, 1970). The possible explanations for the dual responses to isoprenaline (a specific $\beta$-adrenoceptor agonist) may be the presence of fewer $\beta$-inhibitory receptor sites eliciting relaxations. When all these sites are occupied, the drug may activate $\alpha$-adrenoceptors and induce contractile responses.

**Table 2** Incidence of Schultz–Dale reactions in pulmonary blood vessels of the adult chickens

| | *Pulmonary artery* | *Pulmonary vein* |
|---|---|---|
| *Non-sensitized* | | |
| Positive reactors | 0/7 | 0/6 |
| *Sensitized* | | |
| Positive reactors | 15/23 (65%) | 13/17 (76%) |
| I. Strong contractions (50–90% max,) | 5/23 | 4/17 |
| II. Intermediate contractions (25–50% max.) | 7/23 | 5/17 |
| III. Weak contractions (10–25% max.) | 3/23 | 4/17 |

This would indicate relatively poor specificity of isoprenaline on certain blood vessels (Eyre, 1971b; Holroyde & Eyre, 1975; Elmes & Eyre, unpublished) and/or may suggest relatively greater intrinsic activity at $\alpha$-adrenoceptors (Ariens, 1954) in this system.

The insensitivity of avian pulmonary vessels to bradykinin supports similar findings by Somlyo *et al.* (1967). This may at least in part be attributed to chemical differences between the avian kinins (ornithokinins) and other kinins (Eisen, 1971).

The effects of different prostaglandins on isolated vascular smooth muscles have been reported to vary greatly with the species, vascular bed and tone of the vessels (Joiner, Kadowitz, Hughes & Hyman, 1975). Prostaglandin $F_{2\alpha}$ induced dose-dependent contractions of the avian pulmonary vein and artery. Similar contractile responses to prostaglandin $F_{2\alpha}$ have been reported earlier on various blood vessel strips (Hiller & Karim, 1968; Lewis & Eyre, 1972; Burka & Eyre, 1974; Joiner *et al.*, 1975). Low doses of prostaglandin $E_1$ and $E_2$ induced relaxation of avian pulmonary arterial strips irrespective of the spasmogen used; a further increase in the concentrations of $E_1$ and $E_2$ induced contractions of variable magnitudes. Similar biphasic effects have been reported for prostaglandin $E_1$ and $E_2$ on small mesenteric and renal arteries of cats, dogs and rabbit (Strong & Bohr, 1967) and prostaglandin $E_2$ on bovine pulmonary vein (Burka & Eyre, 1974).

Prostaglandin $E_1$ caused strong contractions of pulmonary veins of chickens, while prostaglandin $E_2$ induced reversal of only prostaglandin $F_{2\alpha}$-induced contractions, and produced either no effect or slight contractions of veins partially contracted to other spasmogens.

The pulmonary vessels of the chickens may now be added to the list of vascular tissues showing the Schultz–Dale reaction, e.g. pulmonary vessels of rabbit (Lecomte, 1958), calf (Eyre, 1970; 1971a; 1973; 1975; Eyre & Deline, 1971a, b; 1972), horse (Eyre, 1972; Burka *et al.*, 1976); and the hepatic (Holroyde & Eyre, 1975) and digital (Elmes & Eyre, unpublished) veins of the calf.

The considerable variability in the latent period, duration, magnitude and spontaneity of Schultz–Dale reaction of chicken pulmonary vessels may possibly be atributed to different degrees of hypersensitivity of the tissues to the sensitizing antigen; the number of the mast cells; the amount of vasoactive substances in the mast cells and to physical differences, i.e. proportional distribution of cardiac, circular and longitudinal muscle fibres, amount of the adherent lung tissue. Similar variations were also found with several gut tissues of birds exhibiting the Schultz-Dale reaction (Chand & Eyre, 1976).

In this study the following criteria, characteristic of Schultz–Dale phenomenon, were satisfied: (i) tissue from non-sensitized chickens did not react to specific sensitizing antigen even at high bath concentrations; (ii) tissues from sensitized birds did not react to heterologous or homologous non-specific antigens; (iii) specific antigen-induced contractions were invariably preceded by a latent period of at least 30 s: probably indicative of antigen-antibody reaction and formation and release of vasoactive substances (histamine, 5-HT, prostaglandins, slow reacting substance of anaphylaxis (SRS-A), etc.) from the mast cells; (iv) tachyphylaxis or desensitization to second antigenic challenge, a phenomenon reported earlier by several workers (Kellaway, 1930; Schild *et al.*, 1951; Schild, 1956; Dale & Okpako, 1969; Okpako, 1970, Eyre, 1970; 1971a; Holroyde & Eyre, 1975; Chand & Eyre, 1976); (v) partial recovery of the antigen responses after 1 to 3 h of resensitization 'rest' interval.

Some of sensitized avian pulmonary vessels did not respond to antigen. It has previously been reported that the Schultz–Dale reaction does not always occur in all vascular strips (Eyre, 1970; Eyre & Deline, 1971b; 1972; Holroyde & Eyre, 1975).

Among several possibilities put forward to explain the Schultz–Dale phenomenon the most acceptable hypothesis is the interaction of the antigen with the tissue-fixed antibodies on the mast cells leading to a series of enzymatic reactions resulting in the changes in cell permeability and extrusion (exocytosis) of stored granules of mediators of immediate hypersensitivity and activation of synthesis release of other vasoactive lipids (prostaglandins, RCS, SRS-A, etc.) (Feldberg, 1961; Dale & Zilletti, 1970).

The evidence suggests that *in vitro* anaphylactic contractions of avian pulmonary vessels may not be mediated by histamine. The chicken lung contains few mast cells (Wight, 1970), and these probably contain little histamine (Hunt & Hunt, 1959); chopped lung from sensitized chickens does not release histamine on antigen challenge (Chand, Eyre & Deline, unpublished); $H_1$-receptor blockers and Compound 48/80 do not protect chickens from anaphylaxis (Pedersoli, 1973); and pulmonary vessels are relatively insensitive to histamine. They are, however, more responsive to 5-HT; large amounts of 5-HT (Meyer & Sturkie, 1974) as well as of histamine (El-Acked & Sturkie, 1972) may be released *in vivo* on antigen challenge. Thus, despite insensitivity to histamine, pulmonary vein and arterial constriction resulting in the increase in central venous pressure and consequently right heart dilatation may still occur (Lecomte & Beaumariage, 1958; Aronson *et al.*, 1961).

Nonsteroidal anti-inflammatory drugs (sodium meclofenamate, phenylbutazone), diethylcarbamazine citrate (inhibitor of SRS-A release), disodium cromoglycate, PR-D-92-EA, M & B 22948 (inhibitors of mediator release) are effective inhibitors of central venous pressor responses to antigen in chicken anaphylaxis (Chand & Eyre, unpublished). This may support the involvement of prostaglandins and SRS-A in the reaction of pulmonary vessels to antigen.

There is considerable similarity in the symptoms of anaphylaxis and a disease causing sudden death in growing chickens commonly known as 'flips', 'lung oedema', 'flipover' or 'heart attack' (Howell, 1972; Cassidy, Gibson & Proudfoot, 1975). Histamine and other vasoactive substances may constrict pulmonary capillaries and venules resulting in increased capillary permeability and oedema formation in the lung (Somlyo & Somlyo, 1970). Sudden pulmonary venoconstriction resulting from immediate hypersensitivity reactions may be considered as one of several possible causes of this avian disease of unknown aetilogy (Carlson, personal communication).

The authors wish to thank Prof. H.G. Downie for facilities; Prof. B.S. Reinhart for generous supply of the chickens; Mr T.R. Deline for technical assistance; the Upjohn Company, Kalamazoo, Michigan, for generous gift of the prostaglandins. The work was supported in part by the Ontario Ministry of Agriculture and Food. N. C. is grateful to the Canadian Commonwealth Committee for the award of a Scholarship.

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*(Received July 8, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 209–214

# THE EFFECTS OF RESERPINE AND 6-HYDROXYDOPAMINE ON THE CONCENTRATIONS OF SOME ARYLALKYLAMINES IN RAT BRAIN

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1 The concentrations of *p*- and *m*-tyramine were measured in the caudate nucleus of the rat brain following subcutaneous injection of reserpine or intraventricular injection of 6-hydroxydopamine. $\beta$-Phenylethylamine was analysed in the hypothalamus after reserpine.

2 Endogenous levels of *p*-tyramine and *m*-tyramine in the caudate nucleus, and $\beta$-phenylethylamine in the hypothalamus were 8.02, 2.25 and 2.52 ng/g respectively.

3 Tyramine concentrations were reduced to less than 20% of control values one day after a reserpine injection of 1 or 10 mg/kg. A single dose of reserpine (0.4 mg/kg) significantly decreased the content of both tyramines in the caudate nucleus. The effects became apparent as early as 45 min after drug administration and persisted for at least 6 h in the case of *p*-tyramine and for at least 19 days in the case of *m*-tyramine.

4 The hypothalamic content of $\beta$-phenylethylamine was unaffected by reserpine.

5 Ten days after an intraventricular injection of 6-hydroxydopamine (250 µg), *p*- and *m*-tyramine concentrations in the caudate nucleus were significantly below control levels.

6 The results suggest that *p*- and *m*-tyramine may be stored by an intraneuronal reserpine-sensitive storage mechanism. Alternatively, the tyramines may replace some of the catecholamines from their storage granules and then be released as false transmitters by the nervous impulse. The observed changes in tyramine levels might also reflect the fact that these amines may be metabolically related to another amine which is stored in reserpine-sensitive granules.

## Introduction

$\beta$-Phenylethylamine, *p*-tyramine, *m*-tyramine and tryptamine have been observed in small amounts in the mammalian brain (Saavedra & Axelrod, 1972; Durden, Philips & Boulton, 1973; Philips, Durden & Boulton, 1974a, b; Saavedra, 1974; Willner, LeFevre & Costa, 1974; Boulton, Juorio, Philips & Wu, 1975a; Philips, Davis, Durden & Boulton, 1975) and have been shown to be present in a synaptosomal fraction (Boulton & Baker, 1975). Brain concentrations of the neurotransmitters dopamine, noradrenaline and 5-hydroxytryptamine are known to be severely reduced by reserpine (Shore, Silver & Brodie, 1955; Holzbauer & Vogt, 1956; Bertler, 1961), presumably by impairment of a $Mg^{2+}$-adenosine triphosphate (ATP)-dependent storage mechanism (Carlsson, Hillarp & Waldeck, 1963). More recently it has been shown that reserpine also decreases the level of *p*-tyramine in the optic lobe of *Octopus* (Juorio & Philips, 1975). Administration of 6-hydroxydopamine intravenously to kittens or intraventricularly to mature rats depletes both noradrenaline and dopamine in the brain (Laverty, Sharman & Vogt, 1965; Ungerstedt, 1968; Uretsky & Iversen, 1970; Laverty & Taylor, 1970).

This study examines the effects of reserpine and 6-hydroxydopamine on the concentrations of $\beta$-phenylethylamine, *p*-tyramine and *m*-tyramine in the rat brain. For comparative purposes, the levels of dopamine, noradrenaline and 5-hydroxytryptamine were also measured. Some of the present results have been previously communicated to the Pharmacological Society (Boulton, Juorio, Philips & Wu, 1975b).

## Methods

Male Wistar rats weighing 150–230 g were used. Crystalline reserpine (Sigma Chemical Co., St. Louis, Mo.), the amount depending on the dose to be injected, was dissolved in 0.1–0.2 ml of glacial acetic acid, diluted as required with isotonic glucose solution (at least 50-fold) and injected subcutaneously (2 ml/kg). Control animals were given a corresponding volume of the vehicle. 6-Hydroxydopamine hydrobromide (Sigma Chemical Co., St. Louis, Mo.) was dissolved in cold 0.9% w/v NaCl solution (saline) containing 10 mg/ml of ascorbic acid. Each animal was anaesthetized with sodium pentobarbitone (20 mg/kg, i.p.), and 6-hydroxydopamine (250 µg of the base in 25 µl) was injected into the lateral ventricle of the brain (Noble, Wurtman & Axelrod, 1967). Control animals received an equivalent volume of the vehicle. After appropriate time intervals, the animals were decapitated and the brains quickly dissected into the following parts: the caudate nucleus (including some of the underlying putamen, mean weight 59 mg); the hypothalamus (the tissue below the thalamus and between the anterior and posterior commissura, mean weight 47 mg); the thalamus (mean weight 55 mg); and the 'brain stem' (including the posterior colliculi, the medulla oblongata and the pons to the obex, mean weight 236 mg). After dissection, the tissues were quickly frozen, weighed, and stored on dry ice until analysis (not more than one day).

### *Mass spectrometric analyses*

$\beta$-Phenylethylamine, *p*-tyramine and *m*-tyramine were estimated as their dansyl derivatives by the mass spectrometric integrated ion current technique, with the corresponding deuterated compounds used as internal standards. The caudate nuclei from five animals were pooled for the analysis of *p*- and *m*-tyramine. After weighing, the tissues were homogenized in 5.5 ml of 0.1 N HCl containing 1 mg/ml of disodium edetate (EDTA) and 5 mg/ml of ascorbic acid, and 4 ml was removed for analysis; 1 ml was kept for the estimation of dopamine. The volume was adjusted to approximately 20 ml with 0.4 N perchloric acid, and deuterated *p*- and *m*-tyramine added (25 ng each of the free base). The purity of each deuterated compound was determined by analysing the dansyl derivatives of known amounts of the deuterated compound and the corresponding non-deuterated compound of established purity. By comparison of the areas enclosed by the respective integrated ion current curves (Durden *et al.*, 1973), the amount of deuterated amine actually present in the standard could be determined. A correction factor could be calculated and used to determine the actual amount of deuterated standard added to the tissue samples. An amine fraction was separated and the dansyl derivatives prepared as described previously (Philips *et al.*, 1974a, 1975). The dansylated isomers of *p*- and *m*-tyramine were purified by two successive unidimensional separations on 20 x 20 cm thin layers of silica gel (Brinkmann Instruments Ltd., Rexdale, Ont.) in the solvent systems chloroform : ethyl acetate, 4 : 1 (v/v), and benzene : triethylamine, 12 : 1 (v/v). The dansyl amines were eluted from the silica gel and analysed mass spectrometrically as described earlier (Philips *et al.*, 1974a; 1975).

To analyse $\beta$-phenylethylamine in the hypothalamus, tissue from five animals was pooled, weighed, then homogenized in 2 ml of saturated sodium carbonate containing deuterated phenylethylamine (25 ng of free base). The sample was frozen for 30–60 minutes. After thawing, the amines were extracted into two 1.5 ml portions of acetone and the dansyl derivatives prepared by adding 0.5 ml of dansyl chloride reagent (8 mg/ml in acetone). Following overnight reaction at room temperature, traces of sodium carbonate were precipitated by addition of 10 ml of acetone. The supernatant was transferred to a round bottomed flask and dried by rotary evaporation at 45°C. Dansyl derivates were eluted from the flask with ethyl acetate, reduced to a few drops under a stream of $N_2$, and transferred to a 20 x 20 cm thin layer of silica gel. The dansyl $\beta$-phenylethylamine zone was separated in the solvent system chloroform : ethyl acetate, 4 : 1 (v/v), and further purified on a second layer of silica gel by allowing the solvent system benzene : triethylamine, 8 : 1 (v/v) to rise to a height of 8 cm. The chromatogram was dried, then redeveloped in carbon tetrachloride : triethylamine, 5 : 1 (v/v) to a height of 18 cm. Dansyl $\beta$-phenylethylamine was eluted from the silica gel and analysed mass spectrometrically as previously described (Durden *et al.*, 1973).

### *Fluorimetric analyses*

The catecholamines were estimated by the fluorimetric method proposed by Laverty & Sharman (1965) as described by Juorio (1971). Dopamine estimations were carried out in about one-fifth of the pooled caudate nucleus homogenate, while noradrenaline was estimated in the pooled thalamus obtained from two to five rats. The catecholamines were acetylated and the resulting tri-acetates separated by paper chromatography. The compounds were then eluted and condensed with 1,2-diaminoethane, and the fluorescent products extracted into isobutanol and estimated. 5-Hydroxytryptamine was extracted into butanol from deproteinized tissue extracts at pH 10, and the fluorescence measured in 3 N HCl (Bogdanski, Pletscher, Brodie & Udenfriend, 1956). Pooled 'brain stems' obtained from five rats were used for each 5-hydroxytryptamine estimation. Checks on recoveries of 50–100 ng of added amines were carried out in each experiment; the percentage recoveries were (means ± s.e. mean, number of experiments in brackets) dopamine, 75 ± 4 (12), noradrenaline, 61 ± 4 (10) and 5-hydroxytryptamine, 73 ± 2 (14).

## Results

### *Effect of reserpine*

The concentration of *p*-tyramine in the rat caudate nucleus was approximately 0.07% that of dopamine; *m*-tyramine in the caudate nucleus and $\beta$-phenylethylamine in the hypothalamus were present in even

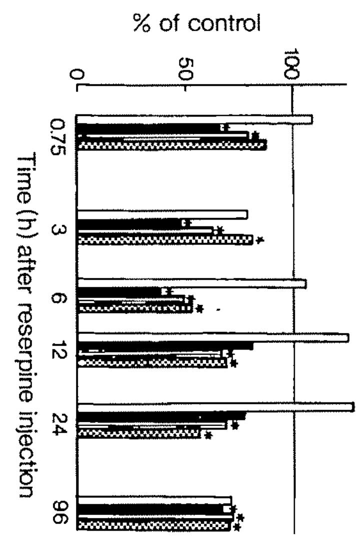

**Figure 1** The effect of reserpine on the concentration of β-phenylethylamine (open columns) in the rat hypothalamus, and *p*-tyramine (solid columns), *m*-tyramine (vertically lined columns) and dopamine (dotted columns) in the rat caudate nucleus. Amine concentrations, expressed as percentages of control values, are plotted against the time (in hours) elapsed between drug administration and the death of the animal. The values are calculated from the results given in Table 1; *: significantly different from control.

smaller amounts (Table 1). Both *p*- and *m*-tyramine were depleted by the subcutaneous administration of reserpine; high doses (1 and 10 mg/kg) reduced the concentrations of these amines to less than 20% of those observed in the solvent-injected controls (Table 1). Similar reductions were observed for dopamine in the caudate nucleus, for noradrenaline in the thalamus, and for 5-hydroxytryptamine in the brain stem. However, the hypothalamic content of β-phenylethylamine was unaffected by these doses of reserpine (Table 1). A lower dose of reserpine had a less pronounced and more selective effect on the brain amine values. One day after the administration of 0.4 mg/kg of reserpine, *m*-tyramine and dopamine in the caudate nucleus, noradrenaline in the thalamus, and 5-hydroxytryptamine in the brain stem were still reduced significantly, but *p*-tyramine in the caudate nucleus was no longer significantly lower than the control value (Table 1). Neither *p*-tyramine nor *m*-tyramine was affected by reserpine at a dose of 0.2 mg/kg, but moderate reductions were observed in dopamine, noradrenaline and 5-hydroxytryptamine values.

The time course of the effect of a single dose of reserpine (0.4 mg/kg) is depicted in Table 1 and Figure 1. A significant decrease in the content of both *p*- and *m*-tyramine was apparent in the caudate nucleus as early as 45 min after administration of the drug, and continued for at least 6 h; after this period *p*-tyramine tended to return to its control value. The concentration of *m*-tyramine, however, remained significantly below the control value for at least 19 days. Dopamine in the caudate nucleus, noradrenaline in the thalamus,

**Table 1** The effect of reserpine on the concentration of β-phenylethylamine (βPE), *p*-tyramine (PTA), *m*-tyramine (MTA), dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in different regions of the rat brain

| *Dose (mg/kg)* | *Time (h)* | *βPE Hypothalamus (ng/g)* | *PTA Caudate nucleus (ng/g)* | *MTA Caudate nucleus (ng/g)* | *DA Caudate nucleus (ng/g)* | *NA Thalamus (ng/g)* | *5-HT 'Brain stem' (ng/g)* |
|---|---|---|---|---|---|---|---|
| Controls | – | 2.52 ± 0.25 (16) | 8.02 ± 0.76 (18) | 2.25 ± 0.08 (15) | 11,069 ± 689 (24) | 615 ± 26 (15) | 463 ± 23 (17) |
| 0.2 | 24 | – | 7.35 ± 0.58 (3) | 2.08 ± 0.12 (3) | 8,245 ± 531 (5)‡ | 464 ± 44 (3)† | 355 ± 44 (3)* |
| 0.4 | 0.75 | 2.75 ± 0.23 (6) | 5.31 ± 0.32 (6)‡ | 1.79 ± 0.16 (6)* | 9,724 ± 1031 (6) | 531 ± 16 (6)* | 408 ± 16 (6) |
| 0.4 | 3 | 1.99 ± 0.15 (3) | 3.81 ± 0.23 (3)§ | 1.57 ± 0.08 (3)§ | 9,048 ± 503 (7)* | 364 ± 23 (7)§ | 336 ± 15 (7)§ |
| 0.4 | 6 | 2.69 ± 0.69 (4) | 3.09 ± 0.29 (4)§ | 1.11 ± 0.05 (4)§ | 5,895 ± 376 (4)§ | 377 ± 43 (4)§ | 374 ± 18 (4)† |
| 0.4 | 12 | 3.17 ± 0.73 (3) | 6.53 ± 0.27 (4) | 1.51 ± 0.06 (4)§ | 7,611 ± 197 (4)§ | 274 ± 15 (4)§ | 278 ± 18 (4)§ |
| 0.4 | 24 | 3.22 ± 0.67 (8) | 6.25 ± 0.78 (8) | 1.56 ± 0.07 (8)§ | 6,372 ± 280 (10)§ | 264 ± 18 (6)§ | 282 ± 18 (8)§ |
| 0.4 | 96 | 1.81 ± 0.28 (4) | 5.42 ± 0.52 (4)* | 1.62 ± 0.07 (4)§ | 7,788 ± 208 (4)§ | 336 ± 16 (4)§ | 274 ± 12 (3)§ |
| 0.4 | 456 | – | 10.50 ± 0.32 (6) | 1.95 ± 0.08 (6)* | 11,029 ± 461 (6) | 462 ± 13 (6)§ | – |
| 1 | 24 | 2.78 ± 0.66 (4) | 1.41 ± 0.29 (7)§ | 0.39 ± 0.10 (8)§ | 1,260 ± 65 (12)§ | 85 ± 13 (11)§ | 115 ± 8 (10)§ |
| 10 | 24 | 5.1;2.6 (2) | 0.64 ± 0.23 (6)§ | 0.21 ± 0.07 (5)§ | 469 ± 33 (10)§ | 39 ± 20 (5)§ | 40 ± 3 (10)§ |

Reserpine was administered subcutaneously. Results are given in ng/g of fresh tissue (± s.e mean, number of determinations in parentheses) and corrected for recoveries.
Student's *t*-test: *$P<0.05$, †$P<0.01$; ‡$P<0.005$, §$P<0.001$.

and 5-hydroxytryptamine in the brain stem, remained low for at least 4 days (Table 1, Figure 1). $\beta$-Phenylethylamine in the hypothalamus was unaffected by reserpine at any of the doses examined.

### *Effect of 6-hydroxydopamine*

Rats that received a single intraventricular dose of 6-hydroxydopamine (250 µg) appeared sedated and lethargic when compared with vehicle-injected controls and showed piloerection. The effect was apparent one day after treatment and continued until the animals were killed on day 10. Spinal convulsions normally produced by decapitation were suppressed by the drug. The concentrations of *p*-tyramine, *m*-tyramine and dopamine in the caudate nucleus, and of noradrenaline in the hypothalamus were reduced significantly below control values by 6-hydroxydopamine; 5-hydroxytryptamine in the brain stem was unaffected (Table 2).

## Discussion

Subcutaneous administration of reserpine elicited reduction in the concentrations of *p*-tyramine, *m*-tyramine, dopamine, noradrenaline and 5-hydroxytryptamine in the rat brain. Carlsson *et al.* (1963) observed that noradrenaline, dopamine and 5-hydroxytryptamine are taken up *in vitro* by bovine adrenal medullary granules by the same $Mg^{2+}$-ATP-dependent mechanism. The uptake is inhibited by reserpine, but the inhibitory effects of the drug may be counteracted by increasing the concentration of catecholamines in the medium. However, tyramine is taken up by a different mechanism which is only slightly activated by $Mg^{2+}$-ATP and is not sensitive to reserpine.

Our present results indicate that in the brain, the *p*- and *m*-isomers of tyramine may be stored by an intraneuronal reserpine-sensitive storage mechanism. This interpretation does not conform with the findings of Carlsson *et al.* (1963). However, their experiments were done *in vitro* on bovine adrenal medullary granules, while the present results were obtained *in vivo* in rat brain. Thus the earlier findings may not necessarily apply to the storage mechanisms for tyramine operating in the rat brain. Furthermore, the low concentrations of *p*-tyramine and *m*-tyramine which have been observed in the rat adrenal gland (10 ng/g and 5 ng/g respectively; Philips & Juorio, unpublished observations), suggest that the adrenals are unlikely to possess an extensive transport system for the tyramines. It could also be that the tyramines replace some of the catecholamines from their storage granules, and are then released by the nervous impulse to act as 'false transmitters' (Dorris & Shore, 1971).

The present results may also reflect the fact that *p*- and *m*-tyramine may be metabolically related to another amine which is stored in reserpine-sensitive granules. Analyses of rat urine have shown that both amines are related metabolically to dopamine (Boulton & Dyck, 1974). However, these studies are indicative of the metabolism of the body as a whole; they may not necessarily reflect the metabolism in the brain. Labelled *m*-tyramine was isolated from rat urine after an intraperitoneal injection of labelled dopamine and DOPA, but only very small amounts of the *para* isomer were found after the injection of dopamine, tyrosine and DOPA as substrates (Boulton & Dyck, 1974). On the other hand, *p*-tyramine does appear to be formed in larger amounts by hydroxylation of $\beta$-phenylethylamine than does *m*-tyramine (Boulton, Dyck & Durden, 1974), and may be synthesized primarily by this route. Thus for *p*-tyramine, at least, it is unlikely that the decrease in amine concentration elicited by reserpine is due to reduced availability of the metabolic precursor, since $\beta$-phenylethylamine is unaffected, at least in the hypothalamus, by reserpine. It seems more likely that *p*-tyramine is stored in a reserpine-sensitive granule. *m*-Tyramine may well be stored in a similar, or even the same compartment, but since it can be synthesized from DOPA and dopamine, and since its resynthesis rate after reserpine is similar to that of dopamine, it seems likely that this amine is associated with the dopamine compartment.

**Table 2** The effect of 6-hydroxydopamine on the concentrations of *p*-tyramine (PTA), *m*-tyramine (MTA), dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in different regions of the rat brain

| *Treatment* | *PTA Caudate nucleus (ng/g)* | *MTA Caudate nucleus (ng/g)* | *DA Caudate nucleus (ng/g)* | *NA Hypothalamus (ng/g)* | *5-HT 'Brain stem' (ng/g)* |
|---|---|---|---|---|---|
| Controls | 10.55 ± 1.89 (5) | 2.07 ± 0.16 (5) | 8439 ± 241 (5) | 1356 ± 171 (3) | 441,455 (2) |
| 6-Hydroxydopamine | 3.57 ± 0.56 (7)* | 0.87 ± 0.12 (7)‡ | 3149 ± 219 (7)‡ | 480 ± 38 (7)† | 408 ± 21 (6) |

Animals treated with 6-hydroxydopamine (250 µg of base) were killed 10 days after a single intraventricular injection of the drug. Results are given in ng/g of fresh tissue (± s.e. mean, number of determinations in parentheses) and corrected for recoveries.
Student's *t*-test: *$P<0.01$; †$P<0.005$; ‡$P<0.001$.

It is somewhat surprising that the octopamines, which are produced metabolically by $\beta$-hydroxylation of the tyramines (Snyder, Glowinski & Axelrod, 1965; Anagnoste & Goldstein, 1967), are not similarly depleted by reserpine, as Harmar & Horn (1976) have reported that in mouse whole brain, octopamine is not reduced significantly 18 h after an intraperitoneal injection of reserpine (5 mg/kg).

The effect of 6-hydroxydopamine on the concentrations of *p*- and *m*-tyramine may also be explained as either a direct effect, in which specific tyraminergic neurones may be destroyed, or as an indirect effect, in which metabolic precursors of the tyramines are depleted by destruction of dopaminergic neurones. From the present experiments it is not possible to differentiate between these various possibilities.

It is interesting to note that the cerebral regional concentrations of dopamine and *p*- and *m*-tyramine do not change in parallel in all instances. Danielson, Wishart & Boulton (1976) have recently shown that 30 min after an injection of (+)-amphetamine sulphate (5 mg/kg), the concentration of *p*-tyramine in the striatum was reduced to about half the control value, while that of *m*-tyramine increased approximately two-fold. Under similar experimental conditions dopamine concentrations increased only slightly (Aylmer, Steinberg & Webster, 1975). These observations may reflect the fact that amphetamine is able to compete with $\beta$-phenylethylamine for the hydroxylating enzyme, thus reducing the synthesis of *p*-tyramine from $\beta$-phenylethylamine, while *m*-tyramine synthesis is enhanced somewhat by the slightly increased concentrations of dopamine.

$\beta$-Phenylethylamine is unaffected by reserpine. Because it is lipid-soluble, it readily crosses the blood-brain barrier (Oldendorf, 1971), and is found in all subcellular fractions of the rat brain, including the synaptosomes (Boulton & Baker, 1975). $\beta$-Phenylethylamine may be stored in reserpine-resistant granules. Alternatively it may not be stored in granules at all, but may exist throughout the cell and be more or less freely exchangeable with extracerebral $\beta$-phenylethylamine. Because of its non-polar nature, and hence its ability to permeate cell membranes, it seems that the latter is more likely. It has been proposed (Boulton, 1976) that $\beta$-phenylethylamine may function as an activator of the synaptic area. As such, it may function to maintain the synapse in a state of activation insufficient to create postsynaptic effects but necessary for propagation of nervous impulses by more conventional neurotransmitters. Such a role would obviate the need for $\beta$-phenylethylamine to be stored in granules or to be otherwise associated with membranous structures.

We thank Dr B.A. Davis for synthesizing the deuterated internal standards, Dr D.A. Durden for supervising the mass spectrometric analyses, and Mr N.F. Binder, Miss E.E. Johnson, Mr H. Miyashita, Mr E.P. Zarycki and Mr W.A. Hunter for expert technical assistance. We are grateful to the Psychiatric Services Branch, Province of Saskatchewan, and the Medical Research Council of Canada for continuing financial support.

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*(Received July 26, 1976)*

*Br. J. Pharmac.* (1977), **59**, 215–217

# CAN BARIUM SUPPORT THE RELEASE OF ACETYLCHOLINE BY NERVE IMPULSES?

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Conventional electrophysiological techniques were used to study the effects of Ba on the release of acetylcholine (ACh) from frog motor nerve terminals. Equimolar substitution of Ba for Ca eliminated end-plate potentials (e.p.ps) without a corresponding decline in the amplitude of the nerve terminal action potential. Miniature end-plate potentials (m.e.p.ps) were readily detectable in Ba solutions despite a depolarized muscle membrane. Studies on the e.p.p in curarized preparations bathed with different concentrations of Ca and Ba suggest that Ba may compete with Ca in the process by which depolarization of the nerve terminal leads to the release of ACh. Repetitive nerve stimulation at 1 Hz in Ba solutions caused 5–20 fold increases in m.e.p.p frequencies (7 experiments). Stimulation of Ba-bathed preparations at 10 Hz elevated m.e.p.p frequencies to very high levels that could not be measured accurately ($\gg$ 100/s). It is suggested that the asynchronous discharge of m.e.p.ps produced by repetitive nerve stimulation is the electrophysiological correlate of the evoked ACh outflow in Ba solutions detected previously by bioassay of the perfusion fluid.

**Introduction** In studies on cat superior cervical ganglion, Ba has been shown to be an effective substitute for Ca in supporting the release of acetylcholine (ACh) by nerve impulses when transmitter output was measured by bioassay of the perfusion fluid (see e.g. Douglas, Lywood & Straub, 1961). These results appear somewhat contradictory to the few reports in the electrophysiological literature which suggest that Ba is an extremely poor substitute for Ca in supporting the evoked release of ACh. For example, the results of Miledi (1966) and Blioch, Glagoleva, Liberman & Nenashev (1968) with frog motor nerve terminals suggest that end-plate potentials (e.p.ps) are only occasionally detectable in Ba solutions, and when produced involve the release of a very small fraction of the quantal output normally observed in Ca solutions. Several reviewers (Hubbard, 1973; Ginsborg & Jenkinson, 1976) have in fact suggested that these small e.p.ps reflect the transient displacement of membrane Ca by Ba (Laskowski & Thies, 1972), Ba in itself being ineffective in the process of evoked ACh release. This study was undertaken in an attempt to resolve this discrepancy and to provide an electrophysiological correlate of the release of ACh into the effluent from stimulated nerve terminals bathed in Ba solutions.

**Methods** The isolated nerve-cutaneous pectoris preparation of the frog was bathed in normal Ringer solution of the following composition (mM): NaCl, 115, KCl 2, $NaHCO_3$ 2 and $CaCl_2$ 1.8. Ba Ringer solution was identical except that 1.8 mM $BaCl_2$ was substituted for $CaCl_2$. Ba-EGTA Ringer solution contained 3 mM $BaCl_2$, 1 mM ethyleneglycol-*bis*(β-aminoethylether)-*N,N'*-tetra-acetic acid (EGTA) and no added Ca, the remaining salts being identical to normal Ringer. Neostigmine methyl sulphate (1–10 μg/ml) and tubocurarine chloride (2.5 μg/ml) were used in some experiments. Solutions were changed by a roller pump (for protocol see Silinsky, 1974). The responses to supramaximal nerve stimulation were recorded intracellularly by conventional methods (Fatt & Katz, 1951). For focal recording of nerve terminal action potentials and e.p.ps, end-plate regions were localized by recording miniature end-plate potentials (m.e.p.ps) extracellularly (Hubbard & Schmidt, 1963). After amplification, signals were fed in parallel into a computer for average transients (Fabritek) and a pen recorder (Brush-Gould). For determining e.p.p. amplitudes, responses to 128 nerve stimuli were averaged and displayed on a storage oscilloscope. M.e.p.p. frequencies were determined from pen records.

**Results** Figure 1a (upper trace) demonstrates that equimolar substitution of Ba for Ca eliminates the e.p.p. without impairing conduction of the action potential into the nerve terminal (n.t.p.). This effect was readily reversible; the e.p.p. reappearing within 5 min after returning the preparation to normal Ca Ringer (Figure 1a, lower trace). The elimination of the e.p.p. in Ba Ringer was not due to an effect of Ba on the ACh-sensitivity of the subsynaptic membrane as m.e.p.ps could be readily observed (Figure 1b) despite the low (40 to 65 mV) resting potential of Ba-bathed muscle (see also Laskowski & Thies, 1972). The addition of Ba to curarized preparations bathed in Ca solutions caused a presynaptic depression of the e.p.p. amplitude which was surmountable by increasing the Ca concentration, suggesting a possible competitive relationship between the two ions. It thus appears that Ba may be an inhibitor of the process by which the action potential causes the synchronous discharge of ACh quanta detectable electrophysiologically as the e.p.p.

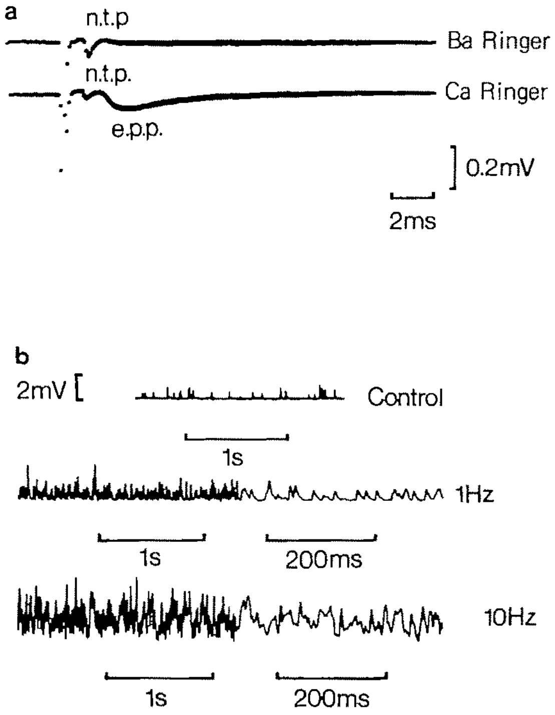

**Figure 1** Effects of Ba on transmission from nerve to muscle. (a) Upper trace – absence of focal end-plate potentials (e.p.ps) after 10 min in Ba Ringer. Identical records were obtained with 1 Hz and 10 Hz stimulation. Lower trace–return of focal e.p.p. (10 Hz stimulation) after 5 min in normal Ca Ringer. n.t.p.-nerve terminal action potential. Each trace represents the averaged response to 128 stimuli. (b) All three traces are from same cell bathed in Ba Ringer with neostigmine. Upper trace – control miniature end-plate potential (m.e.p.p.) frequency (11/s). Middle trace – steady state m.e.p.p. frequency (70-75/s) attained within 1 min after beginning of 1 Hz stimulation. Lower trace – steady state m.e.p.p. frequency ($\gg$100/s) attained within 2 s after beginning 10 Hz stimulation. All 3 traces are photographs of pen records in which stimulus artifacts have been retouched for clarity. Resting potential was 46 mV. Note increased recorder speed for second half of both middle and lower traces.

It should be mentioned that although the disappearance of e.p.ps was a consistent phenomenon, the time course of this effect was quite variable. Generally e.p.ps were eliminated by 60–120 min in Ba but some cells still produced very small e.p.ps for many hours. This variability may be caused by the persistence of small amounts of calcium near certain nerve endings.

If Ba is an antagonist of synchronous quantal release, what then is the source of ACh detected in the perfusion fluid of stimulated cholinergic nerve endings? Although release of ACh from a non-quantal pool cannot be excluded, Figure 1b illustrates a possible electrophysiological correlate for the evoked ACh release, namely an asynchronous residual discharge of m.e.p.ps induced by repetitive nerve stimulation but not phase-locked to the nerve impulse (Braun, Schmidt & Zimmermann, 1966; Miledi & Thies, 1971; Hurlbut, Longnecker & Mauro, 1971). In the experiment illustrated in Figure 1b, the control m.e.p.p. frequency of 11/s (upper record) is increased to a steady-state level of between 70/s and 75/s (middle record) after 1 min of stimulation at 1 Hz and remained at that level for the duration of stimulation (5–6 minutes). The steady-state increase at 1 Hz stimulation ranged from 5 to 20 times the control m.e.p.p. frequency (7 experiments). The maximum evoked frequency often lasted for up to 5–10 s after the stimulation period was terminated. Stimulation at 10 Hz produced a rapid, intense rise of the m.e.p.p. frequency to a maintained level which could not be measured accurately ($\gg$100/s; Figure 1b, lower record). An estimate of this frequency by the method of Katz & Miledi (1972) suggests it to be $\gg$500/second. Rapid unmeasurable increases in m.e.p.p. frequencies were also produced by 10 Hz stimulation after prolonged (6 h) incubation in Ba-EGTA Ringer.

Although increases in m.e.p.p. frequencies are produced by stimulation in Ca solutions, rapid dramatic effects such as those shown in the bottom record of Figure 1b, are not seen at comparable rates of stimulation in Ca solutions (unpublished results; see also Braun *et al.*, 1966, Figure 6). It thus appears that Ba may act directly as an agonist for the asynchronous release of transmitter quanta by repetitive nerve stimulation.

**Discussion** The results suggest that Ba can both antagonize phasic, synchronous ACh release and, in addition, support the process of asynchronous evoked ACh release. The 5–20 fold increase in m.e.p.p. discharges detected in this study under conditions similar to those in the experiments of Douglas *et al.*, (1961) on the superior cervical ganglion (equimolar substitution of Ba for the normal ambient Ca and stimulation at 1 Hz) represent the absolute release of 13 ± 7.75 (mean ± s.e. mean, $n = 7$) *additional* quanta per impulse. Each preganglionic axon in the superior cervical ganglion normally releases $\ll$10 additional quanta per impulse when stimulated at 1 Hz in Ca solutions (Sacchi & Perri, 1973; McLachlan, 1975). It is thus suggested that the asynchronous evoked discharge of m.e.p.ps seen here in Ba Ringer is the electrophysiological correlate of the maintained evoked ACh overflow produced upon substitution of Ba for Ca at the superior cervical ganglion. In this regard similar asynchronous evoked quantal release has been observed at the guinea-pig superior cervical ganglion bathed in Ba solutions by E. M. McLachlan (personal communication), adding considerable support to this contention.

I wish to thank Ms Margaret Silinsky for her photographic expertise. This work was supported by NIH Grant NS12782.

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*(Received September 13, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 218–219

SHORT COMMUNICATION

# STEREOSPECIFICITY OF 2,4-DIAMINOBUTYRIC ACID WITH RESPECT TO INHIBITION OF 4-AMINOBUTYRIC ACID UPTAKE AND BINDING

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S(+)-2,4-Diaminobutyric acid is at least 20 times more potent than the R(—)-stereoisomer as an inhibitor of the sodium-dependent uptake of 4-aminobutyric acid (GABA) in rat brain slices. Both isomers, however, are equipotent as inhibitors of sodium-independent binding of GABA to membranes from rat brain. The latter finding may be relevant to the reported neurotoxicity in rats of both isomers of 2,4-diaminobutyric acid after intracisternal injection.

**Introduction** 2,4-Diaminobutyric acid (DABA) is a neurotoxic amino acid found in various species of *Lathyrus* and *Vicia* (Johnston, 1974) which appears to reduce selectively the sodium-dependent neuronal uptake of the inhibitory transmitter 4-aminobutyric acid (GABA) in slices of rat cerebral cortex (Iversen & Kelly, 1975) and to potentiate the depressant action of GABA on neuronal firing when administered microelectrophoretically (Curtis, Game & Lodge, 1976). The inhibition of GABA uptake by another amino acid, nipecotic acid, is to some degree stereospecific, since R(—)-nipecotic acid is approximately 5 times more potent than the S(+)-isomer (Johnston, Krogsgaard-Larsen, Stephanson & Twitchin, 1976), and earlier experiments with DABA also indicated stereospecificity since the S(+)-isomer was approximately twice as potent as the (±)-racemate (Iversen & Johnston, 1971). We have prepared R(—)-DABA and compared it to commercially available S(+)-DABA with respect to inhibition of sodium-dependent GABA uptake in rat brain slices and to inhibition of sodium-independent binding of GABA to membranes from rat brain.

**Methods** R(—)-DABA was prepared as the dihydrochloride, m.p. 234–237°C, $[\alpha]^{25}_{D} - 19.5°$ (c, 2.0 in water), from R(—)-glutamic acid as described by Adamson (1939) but isolating the product by ion exchange column chromatography. S(+)-DABA monohydrochloride was purchased from Calbiochem, San Diego. The sodium-dependent uptake of GABA ($10^{-8}$ M exogenous concentration) was studied in 'minislices' of rat cerebral cortex at 25°C as described by Beart, Johnston & Uhr (1972). The sodium-independent binding of GABA ($5.8 \times 10^{-9}$ M) to membranes isolated from rat cerebral cortex was studied at 4°C in Tris-citrate buffer as described by Enna & Snyder (1975) using $10^{-3}$ M GABA to correct for nonspecific binding. [2,3-$^3$H]-GABA, specific activity 43 Ci/mmol, was purchased from New England Nuclear, Boston. Percent inhibition values are means ± s.e.m. of quadruplicate experiments.

**Results** R(—)-DABA, $10^{-3}$ M preincubated with the tissue for 15 min, had no significant effect on GABA uptake. This isomer was thus at least 20 times less potent than S(+)-DABA which inhibited GABA uptake under the same conditions by 50% at $5 \times 10^{-5}$M (Iversen & Johnston, 1971). Both isomers inhibited GABA binding to a similar extent; 35 ± 6% inhibition by R(—)-DABA and 32 ± 2% by S(+)-DABA at $10^{-4}$ M. From experiments using inhibitor concentrations of $10^{-4}$ M, $5 \times 10^{-4}$ and $10^{-3}$ M, it was calculated by log-probit analysis (Balcar, Johnston & Stephanson, 1976) that the concentration producing 50% inhibition ($IC_{50}$) of binding was $2.0 \pm 0.4 \times 10^{-4}$ M for R(—)-DABA and $2.3 \pm 0.3 \times 10^{-4}$ M for S(+)-DABA. These results differ from those of Enna & Snyder (1975) who found an $IC_{50}$ of greater than $10^{-3}$ M for DABA, presumably the S(+)-isomer, as an inhibitor of GABA binding, but in the same study the $IC_{50}$ of $2.6 \times 10^{-4}$ M found for DABA inhibition of GABA uptake was appreciably higher than that reported by other workers ($5 \times 10^{-5}$ M, Iversen & Johnston, 1971; $6.6 \times 10^{-5}$M, Harris, Hopkin & Neal, 1973). It is known that S(+)-DABA has a relatively weak depressant action (1/4 to 1/20 as potent as GABA) on the firing of cat spinal neurones (Curtis & Watkins, 1960; Curtis *et al.*, 1976), but this could be due to an indirect effect mediated by release of GABA from presynaptic terminals by heteroexchange as has been suggested for the depolarization of sympathetic ganglion cells by β-alanine (Bowery, Brown, Collins, Galvan, Marsh & Yamini, 1976) since S(+)-DABA appears to be a substrate for the neuronal GABA transport system (Simon & Martin, 1973; Iversen & Kelly, 1975). The present experiments indicate that DABA is some 500 times less active than GABA in displacing radioactive GABA bound to rat brain membranes in the absence of sodium ions.

**Discussion** It has been suggested that the neurotoxic actions of S(+)-DABA may be related to inhibition of GABA uptake (Iversen & Johnston, 1971). This suggestion now seems unlikely since Chen, Flory & Koeppe (1972) observed 'typical neurotoxicity symptoms' in rats after intracisternal injection of *either isomer* of DABA provided the DABA reached a concentration of at least $10^{-3}$ M in the brain. At such concentrations *in vitro* we have noted non-stereospecific inhibitions of sodium-independent GABA binding, a process considered to reflect the binding of GABA to postsynaptic receptors (Enna & Snyder, 1975). It is thus possible that some of the neurotoxic effects of intracisternal DABA are related to interaction with postsynaptic receptors for GABA. Activation of inhibitory receptors might underlie the 'prostrating' effect of intracisternal DABA (Chen *et al.*, 1972) and the 'hypoactivity and sedation' produced by intraperitoneal S(+)-DABA (Shuter & Robins, 1974), but the symptoms of hyper-irritability and convulsions which are delayed in onset (Vivanco, Ramos & Jimenez-Diaz, 1966; Chen *et al.*, 1972) may reflect much more complex events.

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*(Received October 27, 1976.)*

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Printed in Great Britain by The Whitefriars Press Ltd., London and Tonbridge

Volume 59(2)
ISSN 0007-1188
pages 221–378

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February 1977

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# Anabolic-Androgenic Steroids

Editor: C. D. Kochakian

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In recent years the anabolic action of the steroid hormones has become a subject of great interest and activity in both basic and clinical research. In this volume, the editor and his contributors provide a comprehensive survey of the role of the anabolic-androgenic steroids in the vital economy exclusive of androgenic (sexual) functions. Not only does this volume serve as a source of information for investigators in both basic science and clinical research but it also points out and suggests areas for further exploration. Elucidation of the manifold anabolic effects will refine the art of clinical application of these steroids.
Contributors who have been active in the clinical use of these steroids in certain metabolic diseases report their variable results and demarcate areas for further careful trial.

**Contents:**

C. D. Kochakian: Historical Review of Anabolic-Androgenic Steroids.

**Metabolic Effects:** C. D. Kochakian: Metabolic Effects of Anabolic-Androgenic Steroids in Experimental Animals. – R. L. Landau: The Metabolic Effects of Anabolic Steroids in Man.
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**Mechanism of Action:** C. D. Kochakian: Regulation of Protein Synthesis by Anabolic-Androgenic Steroids. – C. D. Kochakian: Regulation of Tissue Enzymes. – C. D. Kochakian, N. Arimasa: The Metabolism in Vitro of Anabolic-Androgenic Steroids by Mammalian Tissues.

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**Appendix:** Chemical Structures and Names of Anabolic-Androgenic Steroids.
Author Index. Subject Index.

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*Br. J. Pharmac.* (1977), 59, 223–229

# MECHANISM OF NEUROTOXICITY OF CARDIOTONIC GLYCOSIDES

B.B. GAITONDÉ & S.N. JOGLEKAR
Haffkine Institute, Parel, Bombay-400 012, India

**1** In cats intracerebroventricular administration of 5, 10, 20 μg of peruvoside, a cardiac glycoside obtained from the plant, *Thevetia neriifolia*, and 10 and 20 μg of ouabain, produced marked neurotoxicity. This was dose-related.

**2** Prior administration of reserpine (2 mg/kg i.m., 500 μg i.c.v.) or tetrabenazine (25 mg/kg i.v., 50 mg/kg i.v. and 2 mg/kg i.c.v.) suppressed the neurotoxicity, but lithium carbonate (100 mg/kg i.p., 2 mg i.c.v.) and haloperidol (200 μg i.c.v.) were ineffective.

**3** Prior administration of 2-bromolysergic acid diethylamide (BOL-148, 200 μg i.c.v.) or *p*-chlorophenylalanine (PCPA) (400 mg/kg i.p.) suppressed the neurotoxicity induced by peruvoside and ouabain.

**4** Perfusion of the lateral ventricles of cats with 10, 20 and 30 μg of peruvoside or ouabain produced a massive release of 5-hydroxytryptamine (5-HT). This was dose-related. Prior administration PCPA suppressed the release of 5-HT.

**5** The results of the findings indicate the involvement of 5-HT in the genesis of neurotoxicity induced by peruvoside or ouabain.

## Introduction

Cardiac glycosides have been reported to produce effects referable to the central nervous system. Gaitondé, McCarthy & Borison (1965), while studying the central emetic mechanism of digitalis glycosides in cats, reported a digitalis toxicity syndrome characterized by emesis, nystagmus, salivation, panting, defaecation, convulsions, hyperpnoea and death. Similar toxic manifestations in cats after administration of peruvoside, a cardiac glycoside obtained from the plant, *Thevetia neriifolia*, have been reported by Gaitondé & Joglekar (1972). Buterbaugh & Spratt (1970) suggested that monoamines might have a role in acute digitoxigenin toxicity. Intracerebroventricular (i.c.v.) injection of digitalis glycosides in minute doses produces marked neurotoxicity (Melville & Shister, 1957; Gaitondé *et al.*, 1965). In this paper we have investigated the mechanism of neurotoxicity induced by cardiac glycosides in cats and elucidated the role of putative neurotransmitters in its genesis.

## Methods

Cats of either sex weighing 3 to 4 kg were used. A cannula was implanted in the lateral cerebral ventricle as described by Feldberg & Sherwood (1954) and experiments were carried out after the cats made a complete recovery (6 to 8 days).

A drug or vehicle was injected i.c.v. in a volume not exceeding 0.25 ml, the effective volume being 0.2 ml. Implantation of the cannula in the cisterna magna of the cat was performed as described by McCarthy & Borison (1966). At the end of each experiment the position of the cannula was verified as described by Feldberg (1963). Those experiments in which the cannula was not in the desired position, were discarded. In all, 110 cats were used in experiments involving i.c.v., i.m. and i.p. injections and 10 cats in experiments involving intracisternal (i.c.) administration.

The severity of the toxic symptoms following administration of cardiotonic glycosides by i.c.v. or i.c. injection in conscious cats was given a score as follows: (a) Each symptom of severe toxicity, as listed in Table 1, received a score of '2'. (b) Mild toxic effects each received a score of '1'. Scoring was done by a 'blind' observer. The total toxicity score for each cat was found by addition. Five cats were used for each dose and the grouped data compared.

The cerebral ventricles of 14 anaesthetized cats were perfused as described by Bhattacharya & Feldberg (1958). The inflow was 0.1 ml per minute. The fluid used for perfusion was artificial

**Table 1** Toxic symptoms following intracerebroventricular administration of various doses of peruvoside or ouabain in conscious cats

| *Severe* | *Mild* |
|---|---|
| Nystagmus | Piloerection |
| Tachypnoea | Defaecation |
| Panting | Micturition |
| Restlessness | Licking |
| Ataxia | Pupillary changes |
| Tremor | Ear flutter |
| Vocalization | Scratching |
| Psychosis | Laboured respiration |
| Rage | Drowsiness |
| Convulsions | |
| Death | |

cerebrospinal fluid prepared as described by Merlis (1940). The effluent was collected in a graduated test tube kept in chilled ice and the volume was recorded every 15 min; the samples were kept at −30°C until assayed for the presence of biogenic substances. Peruvoside or ouabain or 0.9% w/v NaCl solution (saline), was injected into the lateral ventricles via the inflow. The perfusion was stopped for 15 min and the outflow of the polythene tube was closed with a metal cap. This was done in order to allow the full action of the injected drug. At the end of this period, perfusion was restarted and the perfusate collected as described above. Rectal temperature was monitored continuously by a rectal probe (APLAB).

The perfusate was assayed on guinea-pig ileum for acetylcholine and histamine and on rat fundal strip for noradrenaline and 5-hydroxytryptamine (5-HT) (Vane, 1957). The sensitivity of the assays was: 5-HT 2.5 pg/ml, acetylcholine 5 ng/ml, noradrenaline 1 ng/ml, histamine 10 ng/ml.

The following drugs were used: peruvoside (supplied by the Director-General, Indian Council of Medical Research, New Delhi, India), ouabain and 2-bromolysergic acid diethylamide (BOL-148, Sandoz India Ltd., Bombay, India), reserpine (Ciba Research Centre, Bombay, India), tetrabenazine (Roche Pharmaceuticals, Bombay, India) and haloperidol (a gift from Janssen Pharmaceuticals, Belgium). Other drugs were obtained from local commercial firms. A treatment schedule of the cats challenged with peruvoside or ouabain is given in Table 2.

## Results

### *Peruvoside or ouabain-induced neurotoxicity*

Intracerebroventricular administration of 5, 10 and 20 µg of peruvoside or 10 and 20 µg of ouabain produced a sequence of similar toxic effects such as ataxia, nystagmus, panting, piloerection, defaecation, vocalization and death (Table 3). Intracisternally, peruvoside or ouabain produced toxic effects of much less severity. The average toxicity score after

**Table 2** Schedules of drug treatment of cats challenged with peruvoside or ouabain

| *Treatment* | *Dose* | *Route* | *Time of challenge with cardiac glycosides* |
|---|---|---|---|
| Peruvoside | 5, 10, 20, 30 µg | i.c.v. | — |
| Peruvoside | 12.5 µg/kg | i.c. | — |
| Ouabain | 10, 20, 20 µg | i.c.v. | — |
| Ouabain | 6.0 µ/kg | i.c. | — |
| Reserpine | 2 mg/kg | i.m. | 24 hours |
| Reserpine | 500 µg in divided doses | i.c.v. | 4 hours |
| Tetrabenazine | 25 mg/kg | i.v. | 4 hours |
| Tetrabenazine | 50 mg/kg | i.v. | 4 hours |
| Tetrabenazine | 2 mg | i.c.v. | 30 minutes |
| BOL-148 | 200 µg | i.c.v. | 30 minutes |
| Haloperidol | 200 µg | i.c.v. | 30 minutes |
| Lithium carbonate | 100 mg/kg | i.p. | 9th day |
| Lithium carbonate | 2 mg | i.c.v. | 30 minutes |
| PCPA | 400 mg/kg | i.p. | 24 hours |

BOL-148 = 2-bromolysergic acid diethylamide; PCPA = *p*-chlorophenylalanine; i.c.v. = intracerebroventricular; i.m. = intramuscular; i.p. = intraperitoneal; i.c. = intracisternal.

administration of 5 µg of peruvoside i.c.v. was 6.2 ± 0.48 (s.e.); the toxicity score increased three times when the dose of peruvoside was doubled. Further increase in the dose of the drug produced almost maximum toxicity. A dose of 10 µg of ouabain produced a toxicity score of 21.2 ± 1.84 (s.e.) and this was comparable with that produced by 37.5 µg of peruvoside. There was 100% mortality in the group receiving 10 µg of peruvoside or ouabain; the average time required for death to occur was 6 h (range 5–8 hours).

### *Effects of catecholamine-depleting drugs*

Cats pretreated with reserpine or tetrabenazine were challenged with peruvoside. Prior reserpine treatment by the intramuscular or i.c.v. route resulted in a substantial reduction in the toxicity score of almost 50% (Figure 1). A similar effect was noted after treatment with tetrabenazine (Figure 2). Tetrabenazine in an intravenous dose of 25 mg/kg reduced the toxicity score by almost 40% and a higher dose i.e. 50 mg/kg (i.v.) produced a further reduction. Intracerebroventricular tetrabenazine was effective in about one-tenth the intravenous dose.

### *Effect of 2-bromolysergic acid diethylamide (BOL-148) treatment*

Prior i.c.v. administration of BOL-148 in a dose of 200 µg suppressed the toxicity of peruvoside and ouabain (Table 4). The toxicity score was reduced and the incidence of death was substantially lowered.

### *Effects of* p-*chlorophenylalanine (PCPA)*

PCPA in a dose of 400 mg/kg intraperitoneally 24 h before challenge with 20 µg of peruvoside or 10 µg ouabain suppressed the severity of the toxic reaction (Figure 3). There was no mortality in the group of cats receiving PCPA before ouabain or peruvoside challenge, compared with 100% mortality in the untreated group.

**Table 3** Toxicity score in cats after administration of different doses of peruvoside or ouabain

| *Drug* | *Dose (µg i.c.v.)* | *Average score (± s.e.)* | *No. of cats died/tested* |
|---|---|---|---|
| Peruvoside | 5 | 6.2 ± 0.48 | 0/5 |
| | 10 | 18.0 ± 1.14 | 5/5 |
| | 20 | 19.8 ± 0.25 | 5/5 |
| | 37.5 | 21.6 ± 0.12 | 5/5 |
| Ouabain | 10 | 21.2 ± 1.84 | 5/5 |
| | 20 | 27.3 ± 0.66 | 5/5 |

**Table 4** Effect of 2-bromolysergic acid diethylamide (BOL-148) on peruvoside or ouabain-induced toxicity in cats

| *Drug* | *Dose (µg i.c.v.)* | *Average toxicity score (± s.e.)* | |
|---|---|---|---|
| | | *Normal* | *BOL-148* |
| Peruvoside | 20 | 19.8 ± 0.25<br>(5/5) | 6.2 ± 1.14<br>(0/5) |
| Ouabain | 20 | 27.3 ± 0.66<br>(5/5) | 11.0 ± 0.57<br>(1/5) |
| | 10 | 21.2 ± 1.84<br>(5/5) | 5.6 ± 0.34<br>(0/5) |

BOL-148 = 200 µg i.c.v. 30 min before challenge. Figures in parentheses indicate no. of cats died/no. of cats tested.

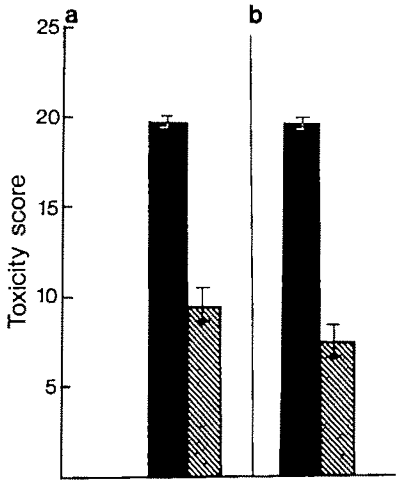

**Figure 1** The effect of reserpine treatment on peruvoside toxicity score in cats. Solid columns—untreated; cross-hatched columns—treated animals (a) Reserpine (2 mg/kg i.m.); (b) reserpine (500 μg i.c.v.).

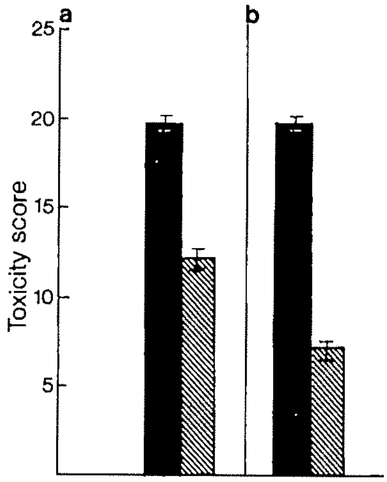

**Figure 2** The effect of tetrabenazine treatment on peruvoside toxicity score in cats. Solid columns—untreated; cross-hatched columns—treated animals (a) Tetrabenazine (25 mg/kg i.v.); (b) tetrabenazine (50 mg/kg i.v.).

*Effects of haloperidol and lithium carbonate*

Prior i.c.v. administration of 200 μg of haloperidol or 2 mg of lithium carbonate failed to suppress the neurotoxicity induced by peruvoside or ouabain (Table 5). Chronic lithium carbonate treatment in a dose of 100 mg/kg (i.p.) for eight days also failed to influence the toxicity. Lithium carbonate (2 mg) in combination with haloperidol (200 μg, i.c.v.) also failed to influence the toxicity of the cardiac glycosides.

*Release of biogenic substances into perfusates of the lateral ventricles*

Injection of peruvoside or ouabain resulted in a detectable release of 5-HT into the perfusate (Figure 4). The greatest amounts of 5-HT appeared in the first 15 min collection period after injection, when the release was dose-dependent. The response to a second injection of peruvoside or ouabain showed tachyphylaxis. Since traces of blood in the perfusate could cause a high 5-HT content we established that no

**Table 5** Effects of haloperidol or lithium carbonate alone or in combination, on peruvoside or ouabain toxicity in cats

| | | *Average toxicity score (± s.e.) after treatment with* | | | | |
|---|---|---|---|---|---|---|
| *Challenge drug* | *Dose (μg i.c.v.)* | *No drug* | *Haloperidol (i.c.v.)* | *Lithium (i.p.)* | *Lithium (i.c.v.)* | *Haloperidol + lithium (i.c.v.)* |
| Peruvoside | 20 | 19.8 ± 0.25<br>(5/5) | 19.0 ± 1.0<br>(5/5) | 19.0 ± 1.0<br>(5/5) | 19.0 ± 2.0<br>(5/5) | 19.0 ± 1.0<br>(5/5) |
| Ouabain | 10 | 21.2 ± 1.8<br>(5/5) | 20.0 ± 1.0<br>(5/5) | 20.0 ± 2.1<br>(5/5) | 19.5 ± 1.1<br>(5/5) | 20.0 ± 1.1<br>(5/5) |

Haloperidol = 200 μg i.c.v.; lithium i.p. = 100 mg/kg i.p. for 9 days; lithium i.c.v. = 2 mg i.c.v. Figures in parentheses indicate no. cats died/no. cats tested.

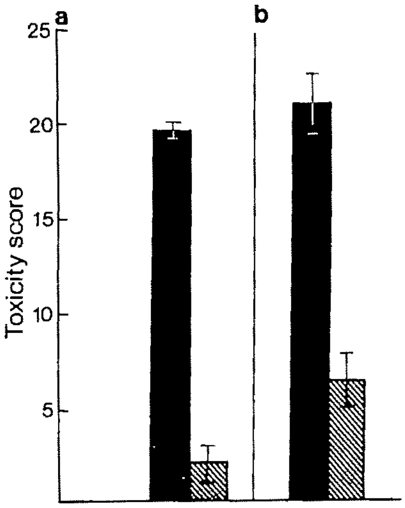

**Figure 3** The effect of *p*-chlorophenylalanine (PCPA) (400 mg/kg i.p.) treatment on peruvoside and ouabain toxicity score in cats. Solid columns—untreated; cross-hatched columns—treated animals. (a) Peruvoside (20 μg i.c.v.) toxicity score; (b) ouabain (10 μg i.c.v.) toxicity score.

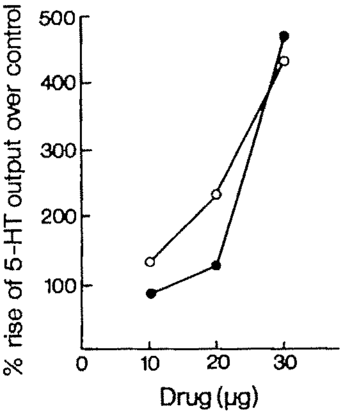

**Figure 4** The rise in 5-hydroxytryptamine (5-HT) output after intracerebroventricular administration of 10, 20 and 30 μg of peruvoside (O) and ouabain (●). The 5-HT content (pg/ml) of the first 15 min collection of perfusate after glycoside injection was expressed as a percentage of that in the 15 min collection immediately before injection. Each point is the mean of 3 experiments.

blood was present by centrifugation and microscopic examination.

Prior administration of PCPA suppressed the 5-HT release induced by peruvoside or ouabain (Figures 5 and 6).

Any histamine, acetylcholine or noradrenaline present in the perfusate was too small in amount to be detected by the assay procedures used.

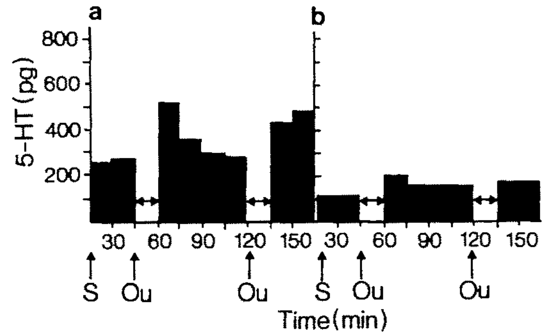

**Figure 5** The release of 5-hydroxytryptamine (5-HT) in the perfusate from lateral ventricles of (a) untreated or (b) *p*-chlorophenylalanine (PCPA)-treated cats. Perfusate was collected every 15 min, except during the 15 min immediately after injection of ouabain (20 μg i.c.v.) when the perfusion was stopped to allow the drug to act (indicated by two-headed arrow). S—saline; Ou—ouabain.

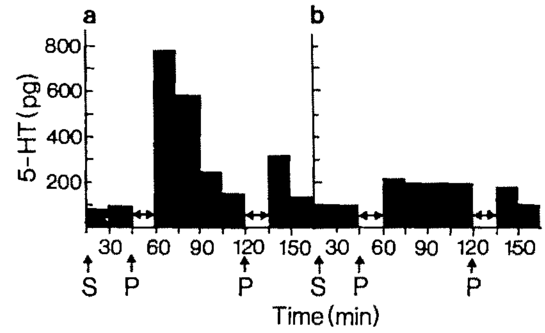

**Figure 6** The release of 5-hydroxytryptamine (5-HT) in the perfusate from lateral ventricles of (a) untreated or (b) *p*-chlorophenylalanine (PCPA)-treated cats. Perfusate was collected every 15 min, except during the 15 min immediately after injection of peruvoside (20 μg i.c.v.) when the perfusion was stopped to allow the drug to act (indicated by two-headed arrow). S—saline; P—peruvoside.

## Discussion

The distribution of a drug introduced into a lateral ventricle of the brain is governed by a number of factors such as volume of injection, concentration of the drug, site of injection, the extent of uptake by periventricular brain tissue or by choroid plexus and the rate of CSF formation. Feldberg (1963) has demonstrated that drugs administered i.c.v. in cats in a volume of 0.25 ml will not only fill the lateral ventricles, but will also fill the 3rd and 4th ventricles and tend to escape into the subarachnoid space more anteriorly and ventrally. Our results indicate that the cardiac glycosides produced a typical 'digitalis toxicity' syndrome. Intracerebroventricular administration of various doses of peruvoside or ouabain produced strikingly severe toxic effects which were dose-related. However, intracisternal administration produced less toxic effects, twitching of the ear and muscles of the neck suggesting a local irritation of the upper cervical segment of the spinal cord.

The neurotoxicity produced by digitalis-like drugs is in all probability due to penetration of these substances from the ependyma into deeper areas of the brain. It is also possible that the ependymal lining may contain chemosensitive receptors which are activated by such drugs and thus reflexly bring about neurotoxicity.

Biologically active substances like catecholamines, 5-HT, histamine and prostaglandins have been postulated to serve as neurotransmitters in specific brain areas by a number of investigators (Vogt, 1954; 1973; Koelle, 1959; Carlsson, 1966; Horton, 1969). Vogt (1954) has shown that administration of various drugs intramuscularly or i.c.v. results in changes in the levels of brain catecholamines. We have found that prior administration of reserpine intramuscularly or i.c.v. suppressed neurotoxicity produced by peruvoside or ouabain. Thus, mortality which is invariably seen after i.c.v. digitalis was almost totally prevented in reserpine-treated animals. Reserpine has been reported to prevent accumulation of catecholamines and 5-HT in the presynaptic storage site (Pletcher, Shore & Brodie, 1955; Shore, Silver & Brodie, 1955; Holzbauer & Vogt, 1956; Muscholl & Vogt, 1958).

Catecholamines or 5-HT, or both, may be involved in the 'digitalis toxicity' syndrome. Our results on cats treated with tetrabenazine also tend to support this hypothesis. The relative role of these two types of transmitters in the genesis of the digitalis toxicity syndrome was further revealed by our studies in animals treated with lithium carbonate. Lithium carbonate has been used therapeutically to suppress 'psychoses' (Cade, 1949; Schoue, 1959; Gershon & Yuwiler, 1960). Since some aspects of digitalis toxicity syndrome resembled 'psychosis', it was decided to investigate the effect of lithium carbonate. The results of the study indicate that digitalis toxicity is unaffected by prior lithium carbonate administration. Stern, Fieve, Neff & Costa (1967) have reported that lithium carbonate stimulates catecholamine turnover in the brain by producing a change in ionic equilibrium at the neuronal membrane. If this is so, our results then indicate catecholamines may not be involved to any large extent in the genesis of digitalis toxicity.

In our previous study we reported that prior administration of haloperidol and pimozide to cats suppressed vomiting induced by peruvoside or ouabain (Gaitondé & Joglekar, 1975). However, administration of haloperidol failed to suppress other neurotoxic effects. Haloperidol has been reported to be a dopamine antagonist (Fuxe & Sjoqvist, 1972). Failure of haloperidol to protect against digitalis toxicity syndrome may be taken to indicate that dopamine may not be involved in its genesis.

BOL-148 (i.c.v.) suppressed considerably the toxicity of digitalis. This drug has been reported to be an antagonist to 5-HT (Garattini & Valzelli, 1965). Buterbaugh & Spratt (1970), while studying the role of brain monoamines in acute digitoxigenin toxicity, suggested that intact brain amine levels have a permissive role in the development of digitoxigenin toxicity, and lethality. In the present study, since BOL-148 suppressed toxicity, we investigated further the possibility of affording protection by pretreating the cats with a drug that reduces the 5-HT concentration in the brain. Our results with PCPA pretreatment clearly demonstrated that reduced brain 5-HT levels suppressed digitalis toxicity. Our findings with BOL-148 and PCPA treatment lend strong support to the hypothesis that the neurotoxicity of digitalis glycosides may be due to the massive release of 5-HT over widespread areas in the brain.

Perfusion experiments in anaesthetized cats in fact do indicate a release of 5-HT into the ventricular perfusate after i.c.v. administration of peruvoside or ouabain. Although the cats were anaesthetized, this release of 5-HT was associated with a number of signs of neurotoxicity such as dilatation of pupils, twitching of facial muscles, ear flutter, movement of the fore legs, defaecation, marked increase in respiration, etc. Rectal temperature showed a drop of almost 1°C. Prior treatment of the cats with PCPA suppressed 5-HT release and also all the associated phenomena of neurotoxicity.

Neurotoxicity becomes one of the problems in therapy with digitalis glycosides. Administration of BOL-148 or a related antagonist to 5-HT may be one of the rational ways of treating digitalis toxicity.

The authors wish to thank The Director, Central Council for Research in Indian Medicine and Homoeopathy, New Delhi, India, for financial assistance.

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*(Received November 14, 1975.*
*Revised August 2, 1976.)*

*Br. J. Pharmac.* (1977), 59, 231–236

# SLEEP INDUCED BY DRUGS INJECTED INTO THE INFERIOR HORN OF THE LATERAL CEREBRAL VENTRICLE IN DOGS

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1 In unanaesthetized dogs, cholinomimetic drugs and their antagonists were injected into the inferior horn of the left lateral cerebral ventricle. Injection volumes of 5 µl were used to limit spread of the drugs beyond the inferior horn. The effects on EEG and behaviour were recorded and compared with the effects of the same doses given into the body of the right lateral ventricle a little behind the foramen of Monro.

2 Injections of cholinomimetic drugs into the inferior horn (acetylcholine 1–2 µg, physostigmine 1.0 µg, pilocarpine 100 µg and nicotine 10 µg) induced sleep during the following hour. The same doses injected into the body of the lateral ventricle did not produce sleep.

3 Cholinolytic drugs (atropine 10–20 µg, hyoscine 0.4–1.6 µg, (±)-tubocuraine 10–20 ng and hexamethonium 40 µg) injected into the inferior horn also produced sleep, but the same doses injected into the body of the lateral ventricle were without effect. The EEG recorded after tubocurarine showed high voltage slow waves during sleep and desynchronized activation during rapid eye movement sleep.

4 Noradrenaline (10 µg) injected into the inferior horn produced sleep whereas the same dose given into the body of lateral ventricle did not produce sleep. The results with 5-hydroxytryptamine were equivocal.

5 It is suggested that the site for induction of sleep lies in structures lining the inferior horn of the lateral cerebral ventricle and that the cholinomimetic drugs probably act by a depolarizing block and the acetlycholine antagonists by a competitive block.

## Introduction

Haranath & Shyamalakumari (1973) described sleep following tubocurarine injected into the lateral ventricle in small doses (500 ng) or perfused in low concentration (10 ng/min) through the cerebral ventricles. They postulated that the site for induction of sleep could be in the structures lining the inferior horn of the lateral cerebral ventricles. Earlier Haranath, Sunanda-bai & Venkatakrishna-Bhatt (1967) had injected cholinomimetic drugs like acetylcholine, physostigmine and neostigmine and their antagonists atropine, tubocuraine and hexamethonium into the carotid arteries of unanaesthetized dogs and observed sleep following both groups of drugs. The present study was designed to establish whether the site of action for sleep induced by the above groups of drugs could indeed be in the structures lining the inferior horn. The effects of noradrenaline and 5-hydroxytryptamine (5-HT) were also studied since they too are involved in sleep mechanisms (Jouvet, 1969).

## Methods

The studies were made on dogs of either sex (6.5–14 kg).

### *Cannulation of the inferior horn and body of the lateral cerebral ventricle*

In dogs under pentobarbitone anaesthesia, a Collison cannula described by Feldberg & Sherwood (1953) and modified by McCarthy & Borison (1966) was screwed into the skull under aseptic conditions 12 mm in front of the zero plane and 17 mm lateral to midline. The shaft length of the cannula was 25 mm so that its tip penetrated the inferior horn of the left lateral ventricle.

Another ventricular cannula, with shaft length 17 mm, was implanted on the opposite side into the body of the right lateral ventricle, a little behind the foramen of Monro (8 mm behind the coronal suture and 5 mm lateral to midline). This position will be referred to as the body of the lateral cerebral ventricle.

### *EEG*

To record the EEG, silver wires were stitched to the skin bilaterally on the frontal, parietal and occipital regions of the head and were connected to an Encardio-Rite polygraph at the time of recording.

### *Observations*

The dogs had recovered completely on the day after implantation of the cannulae and drugs were injected into the cerebral ventricles on the following days. A minute to minute record of the behaviour of the animal was kept. Closure of the eyes for more than 3 min in a sleeping posture with slow and deep respiration and a high voltage slow wave EEG (when recorded) was considered to be sleep. The time until the animal awoke was recorded as a continuous sleep period. Eye movements were seen and were often associated with movements of the muzzle and paws and were confirmed as rapid eye movement (REM) sleep by the EEG changes. The times when these movements occurred were noted in the record. At the effective (sleep-inducing) doses the drug was tried in three or four different dogs and sometimes in the same dog more than once. Only one drug was tried on each day. The animals were generally unrestrained during observations. The dose of a drug necessary to induce sleep was first established without the EEG leads attached to the polygraph. In subsequent experiments with this dose EEG was recorded and mild restraint was necessary at the beginning of the experiment. Soon after the injection the dogs became quiet and later went to sleep when no further restraint was necessary.

Sterilized solutions of drugs were injected into the inferior horn in a volume of 5 µl to limit the spread of the drug only to a small area around the tip of the cannula in the inferior horn. The injections were made with an Agla brand micrometer syringe (Burroughs Wellcome) or Hamilton syringe. For injections into the body of the lateral ventricle the drugs were given in a volume of 100 µl. After completion of all observations methylene blue (0.1%) was injected in the same volume as the drugs into both cannulae and the distribution of the dye was studied *post mortem*. In all the experiments the dye injected into the inferior horn 15 min before the dog was killed was restricted to a 5–10 mm area around the tip of the cannulae and only in a few experiments did it spread up to the genu of the lateral ventricle.

### *Drugs*

Acetylcholine chloride (E. Merck), physostigmine salicylate (T. & H. Smith) pilocarpine nitrate (Boehringer, Ingelheim), nicotine (BDH), atropine sulphate (Merck), (+)-tubocurarine hydrochloride (Koch-Light), hexamethonium tartrate (May and Baker), noradrenaline (Fluka-Buchs) and 5-hydroxytryptamine creatinine sulphate (Merck) were used in these studies. The doses refer to their salts.

## Results

### *Control observations*

Control observations were made for 1 h after injection of 5 µl of 0.85% sodium chloride (saline) into the inferior horn of the lateral cerebral ventricle or for 1 h without any injection. There were no changes of behaviour and no sleep occurred during the first or second hour after saline injection. The observations were made at different times of the day and in different places to exclude conditioning of the responses. If the animal was drowsy during the control observation no drug was injected on that day.

### *Cholinomimetic drugs*

*Acetylcholine* injected into the inferior horn of the lateral cerebral ventricle in doses of 1–2 µg immediately produced ipsilateral ptosis of varying degrees. The dog attempted to wipe its eyes and face with its paws. There was slight salivation. Drowsiness followed in 10–15 min and the animal slept for 3–10 min periods at a time with brief intervals of wakefulness during the following hour. Figure 1 shows the sleep periods from typical experiments after injection of cholinomimetic drugs. Sleep was for longer periods with 2 µg acetylcholine. But with 10 µg acetylcholine the animal showed restlessness, panting and increased depth of respirations but no sleep. When injected into the body of the lateral ventricle, acetylcholine 2 µg did not produce any sleep. It required 20 µg at this site to produce sleep.

*Physostigmine* in doses of 0.01–0.5 µg injected into the inferior horn did not produce any effect. But doses of 1 µg and above produced sleep (Figure 1). Injections of 10 µg physostigmine produced sleep for a long period with frequent twitchings of the muscles of the eyelids, eye balls, muzzle and paws. These are identical with the REM phase of sleep. Feldberg & Sherwood (1954) found cats did not sleep after injection of 10 µg physostigmine into the body of the lateral ventricle. They observed catatonia with large doses.

*Pilocarpine* (100 µg) injected into the inferior horn produced sleep within 10–15 min and each sleep period lasted for 5–10 min at a time with brief intervals of wakefulness. Rapid eye movements were sometimes observed during sleep (Figure 1). In 50 µg doses pilocarpine produced drowsiness, and sleep

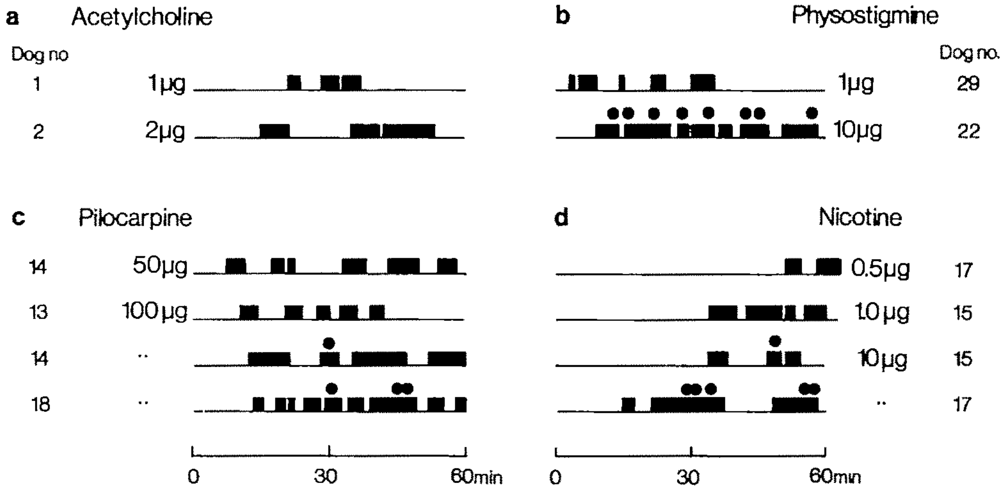

**Figure 1** Sleep periods indicated by horizontal black bars in different experiments. At zero time acetylcholine (a), physostigmine (b), pilocarpine (c) or nicotine (d) was injected into the inferior horn of the lateral cerebral ventricle in conscious dogs, in a 5 µl volume in the doses shown. The black dots over the bars indicate periods of movements of eyes, muzzle and paws during sleep. The numbers given in the margins are the code numbers of the individual dogs.

occurred only in a few instances. In 10 µg doses it produced some salivation and lacrimation but no sleep; 1–2 µg doses had no effect. Pilocarpine 100 µg injected into the body of the lateral ventricle a little behind the foramen of Monro did not produce drowsiness or sleep but only wiping of the face and salivation.

*Nicotine* in doses of 0.05–0.5 µg injected into the inferior horn caused restlessness, retching and increased activity. Doses of 1 µg produced drowsiness and, in some experiments, sleep. The animal wiped its eyes and face on the ipsilateral side for some time. With 10 µg nicotine the animal slept for varying periods after 15 min as can be seen from Figure 1. Rapid eye movements were also observed during sleep. Nicotine 10 µg injected into the body of the lateral ventricle failed to produce sleep or any other effect.

### *Cholinolytic drugs*

*Atropine* 10–20 µg injected into the inferior horn produced immediately increased activity for about 10 min followed by intermittent sleep for nearly 1 h (Figure 2). There was less sleep after 50 µg atropine. Atropine 1 µg was ineffective, while 5 µg produced some drowsiness. In doses of 20–50 µg injected into the body of the lateral ventricle, atropine did not produce sleep.

*Hyoscine* 0.4–1.6 µg injected into the inferior horn produced sleep for varying periods as can be seen from Figure 2. Higher doses of 3.2 µg and lower doses of 0.01–0.2 µg hyoscine did not produce sleep. When injected into the body of the lateral ventricle hyoscine 1 µg produced intense salivation but no sleep. Sleep occurred only with 5–100 µg hyoscine injected into the body of the lateral ventricle. With 1 mg hyoscine injected into the body of the lateral ventricle, the dog defaecated, micturated, became restless and slept after 50 minutes.

*(+)-Tubocurarine* injected into the inferior horn in doses as little as 10–20 ng produced sleep (Figure 2). There were movements of eyes, muzzle and paws during the sleep so produced. Figure 3 shows the EEG taken before and after injection of (+)-tubocurarine 10 ng into the inferior horn. In the record taken 30 min after injection of (+)-tubocurarine the dog was awake and showed increased neuronal activity and desynchronization. After 47 min, the dog was asleep and showed high voltage slow waves. In the 50th min the dog was still asleep but showed rapid eye movements and EEG desynchronization. The same doses of 10–20 ng tubocurarine did not produce any effect when given into the body of the lateral ventricle. The injection of 500 ng (+)-tubocurarine into the body of the lateral cerebral ventricle was required to produce sleep (Haranath & Shyamalakumari, 1973).

*Hexamethonium* 40 µg injected into the inferior horn produced sleep after 10 min and rapid eye movements were frequently observed (Figure 2). No sleep occurred when the dose injected into the inferior horn

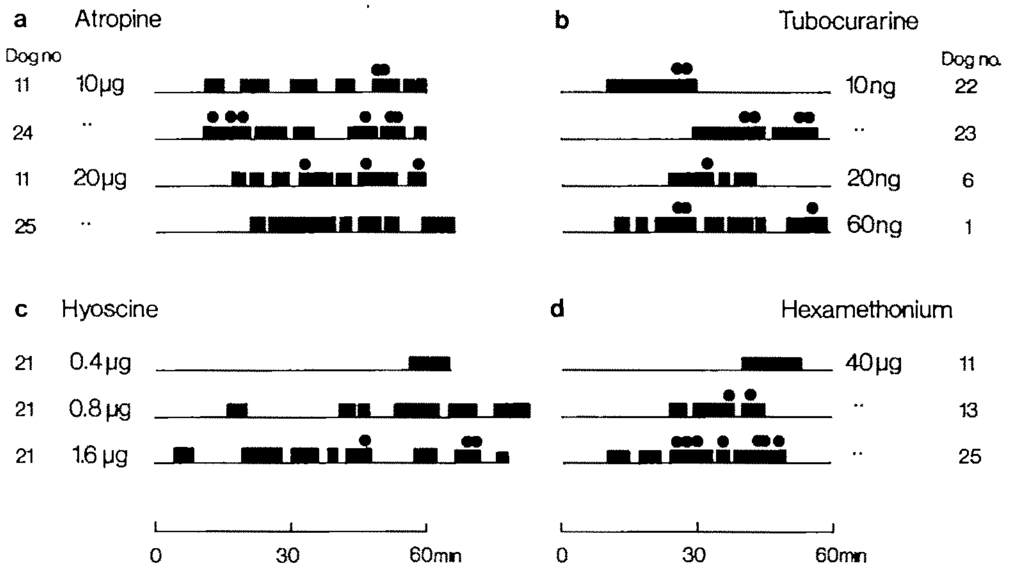

**Figure 2** Sleep periods indicated by horizontal black bars in different experiments. At zero time atropine (a), (+)-tubocurarine (b), hyoscine (c), or hexamethonium (d) was injected into the inferior horn of the lateral cerebral ventricle in conscious dogs in a 5 µl volume in the doses shown. The black dots over the bars indicate periods of movements of eyes, muzzle and paws during sleep. The numbers given in the margins are the code numbers of the individual dogs.

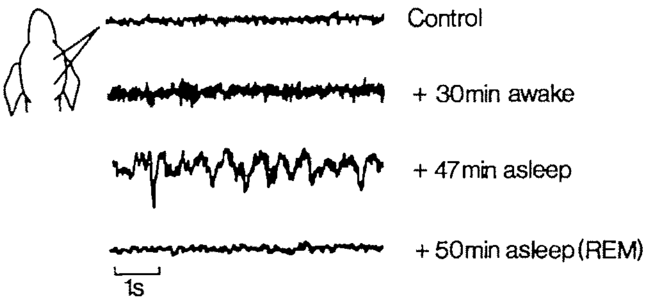

**Figure 3** EEG from an unaesthetized dog. Top record taken before and the rest after injection of (+)-tubocurarine 10 ng into the inferior horn of the left lateral cerebral ventricle. Time of taking the record after the injection and the behavioural state of the animal is given opposite each record.

was reduced to 10 µg, nor when 40 µg hexamethonium was given into the body of the lateral cerebral ventricle.

### *Other drugs*

*Noradrenaline* in doses of 10–20 µg injected into the inferior horn produced sleep lasting for varying periods starting 10–20 min after injection, in only two out of four experiments. But the same dose injected into the body of the lateral ventricle did not produce sleep.

*5-Hydroxytryptamine* injected into the inferior horn in doses of 0.01–0.1 µg produced ptosis and wiping of the face for about 10 min but no sleep. With 0.5 µg 5-HT, sleep occurred after 45 minutes. No sleep occurred when 1 µg 5-HT was given into the body of the lateral ventricle.

## Discussion

The present study supports the suggestion of Haranath & Shyamalakumari (1973) that the site for induction of sleep by small amounts of (+)-tubocurarine injected into the body of the lateral ventricle is in the structures lining the inferior horn. Tubocurarine, even in 10–20 ng doses injected in a 5 µl volume into the inferior horn produced sleep, whereas 500 ng tubocurarine was required to induce sleep when injected into the body of the lateral ventricles. Some idea of the probable extent of the spread of the injected drug can be obtained from the experiments of McCarthy & Borison (1966). They reported that injections of 20 µl volumes of radio-opaque material into the posterior lateral ventricle of a cat, close to the mouth of the inferior horn, outlined only the posterior and inferior portion of the ventricle. Only when 50 µl was injected did the material spread anteriorly. On this basis, in the present experiments the 5 µl injections into the most ventral portion of the inferior horn are unlikely to have spread very far. The distribution of injected methylene blue also supports this conclusion. Thus it appears that the site for induction of sleep by (+)-tubocurarine is in the structures lining the inferior horn. The sleep observed on injection of tubocurarine into the body of the lateral cerebral ventricle could be the result of diffusion into the inferior horn.

That the inferior horn is a site for induction of sleep was borne out not only with (+)-tubocurarine but by the cholinomimetic drugs like acetylcholine, physostigmine, pilocarpine and nicotine, and by the acetylcholine antagonists, atropine, hyoscine and hexamethonium. The above drugs induced sleep in small doses when injected into the inferior horn but the same doses were ineffective when injected into the body of the lateral ventricle.

Since both cholinomimetic drugs and their antagonists produce sleep, this common effect might be achieved through a depolarizing block by the cholinomimetics and a competitive block by the antagonists. The fact that the doses of cholinomimetic drugs required to produce sleep are much higher than those of their antagonists supports this view. This also would fit into the concept of increased activity of cholinergic mechanims in wakefulness and REM sleep. For example, Haranath & Venkatakrishna-Bhatt (1973) reported diminished release of acetylcholine into the perfused cerebral ventricles in unanaesthetized dogs just before and during sleep, and an increased release during REM sleep. Similarly Celesia & Jasper (1966) observed in cats diminished release of acetylcholine from the physostigmine-treated cerebral cortex during natural and barbiturate-induced sleep. Jasper & Tessier (1971) and Gadea-Ciria, Stadler, Lloyd & Bartholini (1973) have also reported diminished release of acetylcholine during slow wave sleep and an increase during REM sleep.

The inferior horn appears to be a site for induction of sleep by other mechanisms also, since noradrenaline induced sleep in much smaller doses when injected into the inferior horn than when given into the body of the lateral ventricle. The results with 5-HT were equivocal since it produced sleep only at a dose of 0.5 µg but not higher or lower doses. Even at that dose, sleep occurred only after 45 minutes.

The important structures lining the inferior horn are the amygdala and the hippocampus. Although it is unlikely that a single neurohumoral mechanism can be solely responsible for induction of sleep at all levels (King, 1971), it is likely that cholinergic mechanisms are involved in some pathway situated in the limbic system (Hernandez-Peon, Chavez-Ibarra, Morgane & Timo-Iaria, 1963) and particularly in the hippocampal region (Kim, Choi, Kim, Kim, Kim, Park & Ahn, 1975).

This work was carried out with a grant-in-aid from the Indian Council of Medical Research, which is gratefully acknowledged.

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*Br. J. Pharmac.* (1977), 59, 237–242

# FREQUENCY-DEPENDENT CHANGES IN THE CARDIAC SARCOLEMMAL ATPASE

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1 Effects of various frequencies (0.25, 0.5, 1.0, 1.5 or 2.0 Hz) of stimulation for various durations (2, 5, 10 or 15 min) on the contractile force of trabecular or papillary muscles of dog myocardium were investigated.

2 Effects of various frequencies (0, 0.25, 0.5, 1.0, 2.0 Hz) of various stimulus strengths (0.5, 1, 10 V) for various durations (2, 5, 10 or 15 min) on the $Mg^{2+}$-dependent $Na^+$-$K^+$-adenosinetriphosphatase (ATPase) of isolated sarcolemmal fraction of dog myocardium were determined.

3 There was a frequency-dependent increase in the contractility and inhibition of the $Na^+$-$K^+$-ATPase within 2 minutes.

4 Frequency-dependent increase in the contractility and inhibition of $Na^+$-$K^+$-ATPase decreased as the duration of stimulation was increased.

5 The diminution in the inhibition of ATPase was associated with a decrease in the contractility with prolonged stimulation.

6 These results suggest that the frequency-dependent increase in the myocardial contractility might be mediated through an inhibition of the sarcolemmal ATPase.

## Introduction

Frequency-dependent changes in the force of contraction have been reported both *in situ* (Lendrum, Boyd & Katz, 1960) and in isolated tissue preparations (Blinks & Koch-Weser, 1961; Katzung & Scheider, 1957). Striking similarities between the positive inotropic effect produced by cardiac glycosides and that produced by changes in the frequency of contraction have also been demonstrated. Thus, as with cardiac glycosides, an increase in frequency of stimulation has been reported to produce an increase in the rate of tension development and a reduction in time to peak tension (Abbot & Mommerts, 1959; Blinks & Koch-Weser, 1961; Koch-Weser, 1963; Reiter & Strickel, 1968). Since it has been suggested that cardiac glycosides produce their positive inotropic effect by inhibiting $Mg^{2+}$-dependent $Na^+$-$K^+$-stimulated membrane adenosinetriphosphatase (ATPase) (Prasad & Callaghan, 1969; Akera, Larsen & Brody, 1970; Besch, Allen, Glick & Schwartz, 1970; Prasad, 1970; 1972; 1974; 1975), it is possible that the positive inotropic effect of increasing the frequency of stimulation might be mediated through an inhibition of the membrane ATPase of the myocardium. If the increase in the force of contraction is related to the inhibition of $Na^+$-$K^+$-ATPase, the possibility exists that this inhibition of $Na^+$-$K^+$-ATPase is frequency-dependent. Indeed preliminary data of Prasad & Kidwai (1974) do indicate that this may be so.

It was therefore decided to study in detail, the effects of frequency of stimulation on $Mg^{2+}$-dependent $Na^+$-$K^+$-stimulated sarcolemmal ATPase of isolated membrane fraction from the dog ventricle.

## Methods

Dogs of either sex weighing between 16–20 kg were anaesthetized with pentobarbitone sodium 35 mg/kg intravenously. The chest was opened through the left fifth intercostal space and the heart was exposed. The pericardium was removed and 5 to 10 g of the left or right ventricle was removed and placed in a cold (–4°C) 0.25 M sucrose solution prepared in glass distilled water. Relatively pure sarcolemma from cardiac muscle was prepared by the method of Kidwai, Radcliffe, Duchon & Daniel (1971) and the $Mg^{2+}$-dependent $Na^+$-$K^+$stimulated ATPase was determined. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Protein determinations were performed before ATPase activity measurements so that equivalent amounts of protein could be used for study. The total volume of enzyme assay system was 1.0 ml and contained

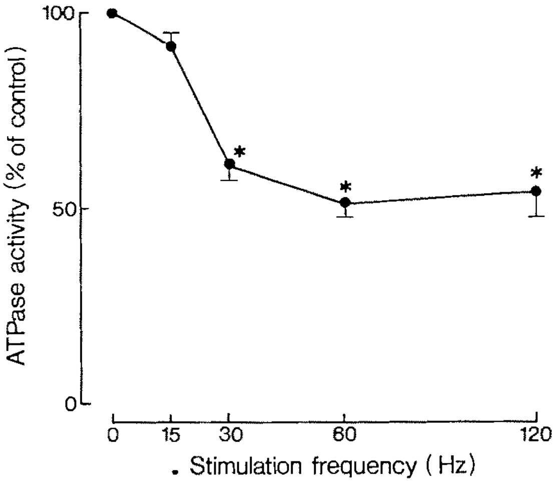

**Figure 1** Effects of various frequencies of sarcolemmal stimulation on sarcolemmal $Na^+$-$K^+$-ATPase in dog heart; stimulus strengths 0.5 V, duration 2 minutes. The results are expressed as percentage of control (without stimulation but incubated for 2 minutes). Each point on the curve is the mean value ± s.e. (bar) from 6 experiments. * Indicates significant difference from control ($P < 0.05$).

(mmol/l): $MgCl_2$ 4, NaCl 50, KCl 4, Tris ATP 4, L-histidine 50 and 50 µg of sarcolemmal protein. The reaction mixture (the enzyme assay system minus Tris ATP) was placed in special tubes, fitted at the base with platinum electrodes such that both the electrodes were submerged in the mixture medium. The electrodes were connected to the Grass stimulator (Model SD9) for stimulation of the mixture media. The tubes containing the reaction mixture was placed in a constant temperature water bath (Dubnoff Metabolic Shaking Incubator, Precision Scientific Corporation) at 37°C. The temperature was maintained at 37°C. The reaction was started by addition of ATP and terminated by addition of 1.0 ml of 10% trichloracetic acid. Inorganic phosphate was determined by the method of Fiske & SubbaRow (1925). The results are expressed as µmol of inorganic phosphate liberated $mg^{-1}$ protein $h^{-1}$. Adenosinetriphosphatase activity assayed in the presence of ouabain 1 mmol/l was subtracted from total activity assayed in the absence of ouabain. This value represented the $Mg^{2+}$-dependent $Na^+$-$K^+$-stimulated ouabain-sensitive portion of ATPase activity. Similarly $Na^+$-$K^+$-ATPase in the presence of various frequencies of stimulation was determined.

For electrical stimulation of the isolated sarcolemmal fraction, the reaction mixture without ATP was placed in a special tube, fitted at the base with platinum electrodes such that both the electrodes were submerged in the mixture medium. The electrodes were connected to the Grass Model SD9 stimulator for stimulation of the mixture media at different frequencies. The tubes containing reaction mixture were placed in the constant temperature water bath (Dubnoff Metabolic Shaking Incubator) at 37°C. The mixtures were incubated at 37°C and were stimulated at various frequencies and voltages for various times. Addition of the ATP to and stimulation of the mixtures were carried out simultaneously. The stimulation of the mixture was carried out at four different frequencies (0.25, 0.5, 1.0 and 2.0 Hz) and stimulus strength of 0.5, 1 and 10 V each for a period of 2, 5, 10 and 15 minutes. The duration of each impulse was 5 ms in all cases. At the end of the experiment the reaction was terminated by addition of 1 ml of 10% trichloracetic acid (TCA).

Trabeculae carnae or papillary muscles were removed from the right or left ventricle of the same dog from which the muscles were removed for isolation of membrane fraction. The set-up for recording the contractility of the muscle was the same as used by Prasad (1975). The thickness of the muscles used in this experiment was such that there was no central core of hypoxia.

## Results

### *Stimulation frequency and ATPase*

Experiments were conducted in which reaction mixtures containing sarcolemmal fraction incubated at 37°C were stimulated at frequencies of 0, 0.25, 0.5, 1.0 or 2.0 Hz with a stimulus strength of 0.5 V for a duration of 2 min and the sarcolemmal ATPase was determined for each frequency of stimulation. The results are summarized in Figure 1. The sarcolemmal ATPase for the unstimulated fraction incubated for 2 min was found to be 13.39 ± 0.9 µmol $mg^{-1}$ protein $h^{-1}$. The sarcolemmal ATPase was found to be inhibited at all frequencies of stimulation, the inhibition being directly proportional to the rate of stimulation from 0 to 1.0 Hz. A further increase in the frequency of stimulation to 2.0 Hz did not produce any further inhibition of sarcolemmal ATPase, on the other hand there was a tendency for a decrease in the inhibition.

### *Stimulation frequency and contractile force*

In five experiments the trabecular muscles were stimulated at various frequencies (0.5, 1.0, 1.5 or 2.0 Hz) for various lengths of time (2, 5, 10 or 15 min) and the changes in the contractile force were recorded. Figure 2 is a representative recording from one trabecular muscle. It is apparent from the tracing that the increase in the contractile force was frequency-

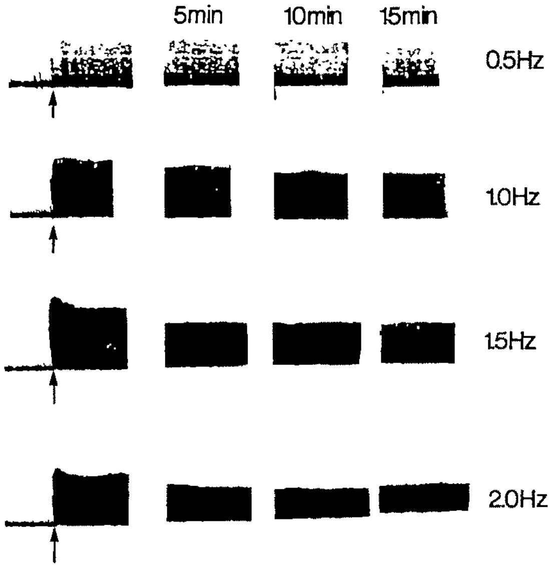

**Figure 2** Effects of different rates of stimulation for different periods on the contractility of trabecular muscle of dog heart. The muscles were stimulated in the control period at the rate of 0.25 Hz. Arrows mark the beginning of the stimulation of the muscles at different frequencies given at the end of each strip of tracing. The time at the top of the tracings indicate the time when that strip was recorded.

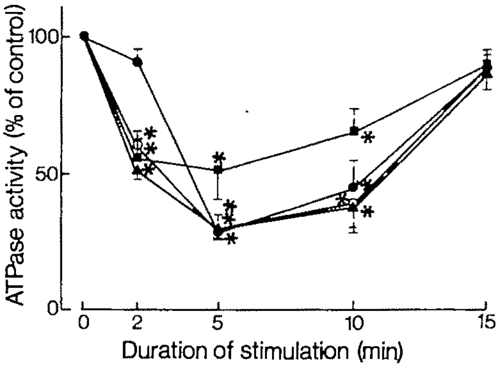

**Figure 3** Effects of different frequencies of stimulation for various periods (2, 5, 10 or 15 min) on sarcolemmal ATPase of dog heart; the results are expressed as percentage of control (without stimulation but incubated for 2, 5, 10 or 15 minutes). Stimulation frequency: (●) 0.25, (O) 0.5, (▲) 1.0 and (■) 2.0 Hz, stimulus strength 0.5 V. Each point on the curve is the mean value from 6 experiments. Vertical lines show s.e. mean. The value at 0 along the duration axis denotes the value of ATPase for different incubation periods without stimulation (control). * indicates that the changes are significant ($P < 0.05$).

dependent initially but with stimulation for 5 min and longer the increase in the contractility was inversely related to the rate of stimulation. At 15 min the contractility was even less than control when stimulated at a rate of 2.0 Hz. These results indicate that high rates of stimulation for prolonged periods did not increase the contractility as they did initially.

### *Stimulation duration and ATPase*

The preceding experiment showed that long periods of stimulation at different frequencies affected the contractility adversely. This decline in the increase of contractility with increased duration might be due to less inhibition of the ATPase and/or decrease in the amount of available energy for contraction. To see if a decrease in the inhibition of ATPase with increased duration for different frequencies might be a factor for reduction in the increase in contractility, experiments were conducted in which the mixtures containing sarcolemmal fraction were stimulated at different frequencies with stimulus strength of 0.5 V for various periods (2, 5, 10, or 15 min) and the sarcolemmal ATPase was determined. The results are summarized in Figure 3. The sarcolemmal ATPase activity without stimulation was found to be 13.4 ± 0.9, 12.15 ± 0.28, 11.1 ± 0.46, 11.43 ± 0.79 μmol $mg^{-1}$ protein $h^{-1}$ respectively for 2, 5, 10 and 15 min duration of incubation without stimulation. An increase in the duration of stimulation from 2 to 5 min caused an increased inhibition of ATPase at all frequencies. A further increase in the duration of stimulation caused less inhibition of ATPase than with a shorter duration. At 15 min, the ATPase activity tended to approach the control value. At a frequency of 2 Hz the inhibition of ATPase was less than that with lower frequencies. These results indicate that higher frequencies and longer duration, produced less inhibition of the sarcolemmal ATPase.

### *Stimulus strength and ATPase*

The above results suggested that the heat produced by the increase in the frequency or duration of stimulation might produce less inhibition. If this is the case then an increase in the strength of stimulation at those frequencies and duration might produce more heat and hence would produce still less inhibition of ATPase. To test this assumption eight experiments were carried out in which reaction mixtures containing sarcolemmal fraction were stimulated at various frequencies with a stimulus strength of 1 or 10 V for various durations and the sarcolemmal ATPase was determined in each case. The results are summarized

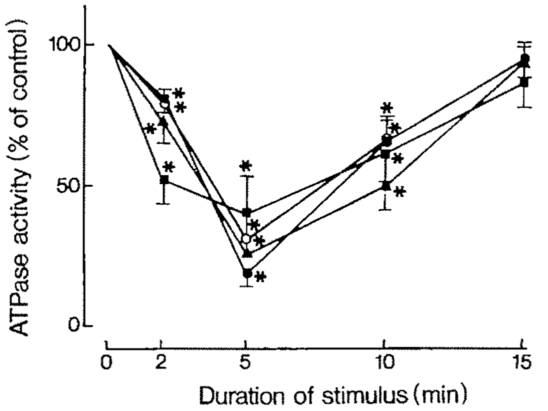

**Figure 4** Effect of stimulation of mixture containing sarcolemmal fraction on sarcolemmal ATPase of dog heart at various frequencies and durations with a stimulus strength of 1 Volt. Frequencies: (●) 0.25; (○) 0.5; (▲) 1.0 and (■) 2.0 Hz. Each point on the curves is mean from 8 experiments. Vertical lines show s.e. mean. * Indicates the changes are significant ($P < 0.05$).

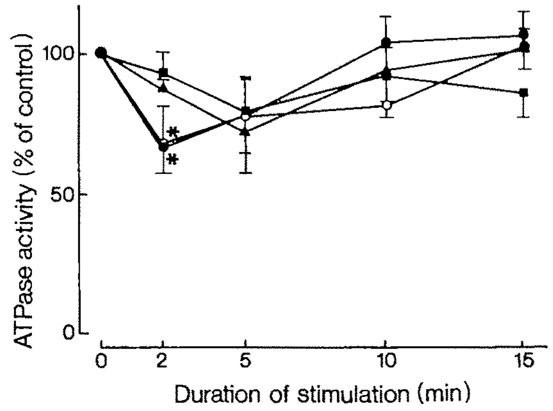

**Figure 5** Effects of stimulation of reaction mixtures containing sarcolemmal fraction on the sarcolemmal ATPase of dog heart at various frequencies and durations with a stimulus strength of 10 Volts. Frequencies: (●) 0.25; (○) 0.5; (▲) 1.0 and (■) 2.0 Hz. Each point on the curves is the mean from 8 experiments. Vertical lines show s.e. mean. * Indicates the changes are significant ($P < 0.05$).

in Figures 3, 4 and 5. An increase in the stimulus strength from 0.5 to 1.0 V caused slightly more inhibition of ATPase at all frequencies of stimulation. However, when stimulus strength was increased to 10 V there was no significant inhibition of the ATPase at any frequencies and durations except at 2 min with frequencies of 0.25 and 0.5 Hz. At 10 and 15 min duration of stimulation the ATPase was almost normal or more than control value.

### *Effects of stimulation on temperature changes in the reaction mixture*

Since the sarcolemmal $Na^+$-$K^+$-ATPase is affected by changes in temperature (Charnock & Post, 1963) and since the electrical stimulation of the reaction mixture might raise the temperature of the reaction mixture, it was decided to see if there was a change in the temperature in the reaction mixture due to electrical stimulation.

Effects of different frequencies (0.25, 0.5, 1.0 and 2.0 Hz) of stimulus strength of 0.5, 1 and 10 V for duration of 2, 5, 10 and 15 min were studied on the temperature changes in the reaction mixture incubated in the constant temperature water bath (Dubnoff Metabolic Shaking Incubator) at 37°C. The probe of a Thermistor Bridge (E & M Instrument Co. Inc., Houston, Texas) was placed in the reaction mixture and the temperature was continuously recorded on the Physiograph MKIII. The temperature changes were recorded with and without stimulation of the reaction mixture. There were phasic changes in the temperature, the maximum change being 0.44°C (Figure 4). This phasic change was associated with the off and on of the heater of the Dufnoff metabolic shaker. Stimulation of the reaction mixture at all frequencies and stimulus strength for all durations did not change the temperature. The phasic change in the temperature associated with the 'off' and 'on' of the heater of the Dufnoff Metabolic Shaker was present during the stimulation of the reaction mixture and this phasic change was never more than 0.44°C. A typical tracing with maximum voltage (10 V) and frequency (2.0 Hz) for 15 min is shown in Figure 6. It is thus apparent that the electrical stimulation of the reaction mixture in a temperature-controlled water bath did not produce any significant change in the temperature of the reaction mixture.

## Discussion

The results indicate that there is a frequency-dependent increase in the contractility and an inhibition of sarcolemmal $Na^+$-$K^+$-ATPase. However, an increase in the duration of stimulation or an increase in the strength of stimulus at different frequencies produced a decrease in the inhibition of sarcolemmal ATPase. Also the frequency-dependent increase in the contractility decreased when the duration of stimulation was increased.

The increase in cardiac contractility with an increase in the frequency of stimulation is well known (Katzung, Rasin & Scheider, 1957; Lendrum, Feinberg, Boyd & Katz, 1960; Blinks & Koch-Weser, 1961). The present results confirm our (Prasad &

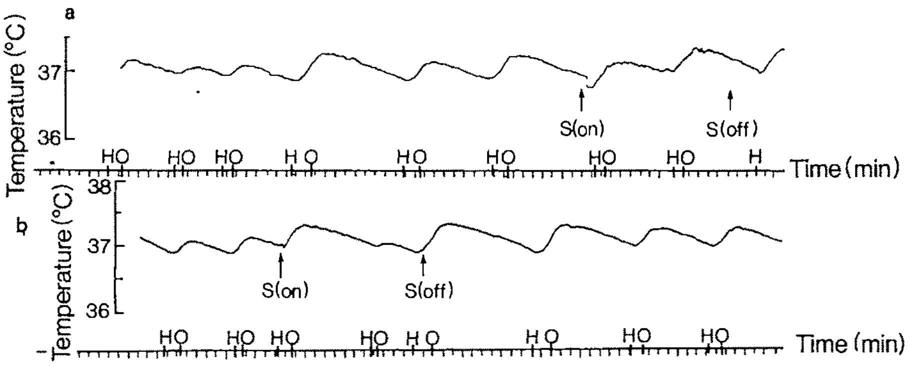

**Figure 6** Effects of electrical stimulation on the temperature changes in reaction mixture. In (a) temperature was recorded for 50 min before electrical stimulation started. In (b) temperature was recorded before and after electrical stimulation. H, pilot light of Dubnoff Metabolic Shaker is on when the temperature starts falling; O, pilot light of the heater off when the temperature starts rising; S, stimulation of reaction mixture at the rate of 2.0 Hz with a stimulus strength of 10 V of 5 ms duration. At the arrow S(On) the stimulation of the reaction mixture begins and at arrow S(Off) the stimulation ends. The rest of the tracing is of the temperature changes in reaction mixture without stimulation. Each mark on the time scale represents one minute. Note that the electrical stimulation of the mixture did not produce any significant change in the temperature of the reaction mixture.

Kidwai, 1974) preliminary observation that electrical stimulation of isolated sarcolemmal fraction produces an inhibition of the sarcolemmal ATPase. The increase in the frequency from 0.25 to 0.5, 1.0, 1.5 or 2.0 produced an increase in the contractility that was inversely related to the rate of stimulation when the duration of stimulation was increased. Similarly the increase in the rate of stimulation produced an inhibition of the sarcolemmal $Na^+$-$K^+$-ATPase which was inversely related to the rate of stimulation when the duration of stimulation was increased. Thus it appears that the frequency-dependent increase in contractility might be related to an inhibition of the sarcolemmal $Na^+$-$K^+$-ATPase. It has been observed, in our laboratory (unpublished results), that agents which inhibit membrane ATPase either reduce or completely abolish the rate-dependent increase in contractility. However, the frequency-dependent increase in contractility decreased at very high frequency (2.0 Hz) and when the duration of stimulation was increased. This could be possible if there were a decrease in the inhibition of the sarcolemmal ATPase and/or a decrease in the available energy at higher frequencies and prolonged stimulation. Certainly these results show that there was a decrease in the inhibition of the sarcolemmal $Na^+$-$K^+$-ATPase at higher frequencies and prolonged stimulation. The question of available energy is not considered in the present paper. The question arises why there should be a decrease in the inhibition of $Na^+$-$K^+$-ATPase at very high frequency or prolonged stimulation. The decrease in the inhibition of sarcolemmal $Na^+$-$K^+$-ATPase with prolonged stimulation at various frequencies might be due to the fact that electrical stimulation of the reaction mixture containing sarcolemmal fraction might be producing enough heat to raise the temperature of the reaction media which in turn might stimulate the sarcolemmal $Na^+$-$K^+$-ATPase. An increase in temperature has been reported to increase the $Mg^{2+}$-dependent, $Na^+$-$K^+$-stimulated microsomal ATPase (Charnock & Post, 1963; Emmelot & Bos, 1966). However, the present results show that there was no significant change in temperature of the reaction mixture due to electrical stimulation possibly because of the controlled temperature water bath. At present no plausible explanation for less inhibition of $Na^+$-$K^+$-ATPase at higher frequencies or with prolonged stimulation or at higher voltages of stimulation can be offered.

The question now arises how this inhibition of $Na^+$-$K^+$-ATPase would increase myocardial contractility. Several models have been proposed for ionic movement in cardiac muscle (Langer, 1971; Prasad, 1974). These models suggest that sarcolemmal $Na^+$-$K^+$-ATPase is not only involved in the efflux and influx of $Na^+$ and $K^+$ but also in influx and efflux of $Ca^{2+}$. An inhibition of sarcolemmal $Na^+$-$K^+$-ATPase has been suggested to increase $Ca^{2+}$ influx (Prasad, 1970; 1974). The present results indicate that there is a frequency-dependent inhibition of the sarcolemmal ATPase. This inhibition of ATPase would increase the $Ca^{2+}$ influx and hence the contractility. Thus, the results suggest that frequency-dependent increase in contractility might be mediated through an inhibition of the sarcolemmal $Na^+$-$K^+$-ATPase.

This work was supported by the Saskatchewan Heart Foundation.

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*(Received March 16, 1976.
Revised August 12, 1976.)*

*Br. J. Pharmac.* (1977), 59, 243–246

# VASCULAR REACTIVITY OF PERFUSED VASCULAR BED IN SPONTANEOUSLY HYPERTENSIVE AND NORMOTENSIVE RATS

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1 Hypertensive and normotensive rats of the same age group were isolated from an inbred colony of spontaneously hypertensive rats.

2 The perfused hindquarter and mesenteric artery preparations obtained from hypertensive and normotensive rats exhibited an increased reactivity to noradrenaline (NA) and angiotensin II.

3 Dose-response curves to NA obtained from hypertensive and normotensive rats exhibited a steeper slope and higher maximum than those from control rats.

4 These findings suggest that increased vascular reactivity of blood vessels is independent of the development or maintenance of elevated blood pressure.

## Introduction

Vascular reactivity to different pressor agents has been extensively studied in different types of experimental hypertension in animals, either in the whole animal preparation (Folkow, Hällback, Lundgren & Weiss, 1970a) or in isolated organ preparations (Folkow, Hällback, Lundgren & Weiss, 1970b; Haeusler & Finch, 1972). The underlying mechanism for this hyper-responsiveness and its role in the development of hypertension is not clear. However, it has been suggested that elevated blood pressure may induce adaptive structural changes in the vessel walls, resulting in an increased wall/lumen ratio. This might be responsible for increased vascular reactivity to vasoconstrictor stimuli (Folkow, Grimby & Thulesius, 1958).

Recently it has been shown that in hypertensive rats the increased reactivity to vasoconstrictor agents persisted even after the blood pressure had been normalized by antihypertensive treatment (Finch, 1974; 1975; Wayyes & Paterson, 1975). These authors have suggested that adaptive/structural changes are not entirely responsible for the increased reactivity of the blood vessels and that hyper-responsiveness is only secondary to hypertension.

In our laboratory we have succeeded in isolating rats from an inbred colony of spontaneously hypertensive rats differing in their blood pressure and which are classified as hypertensive (SH) and normotensive (NSH). These rats were used for investigating the relationship between blood pressure levels and vascular reactivity to noradrenaline (NA) and angiotensin II in vascularly isolated but neurologically intact hindquarter and mesenteric artery preparations.

## Methods

Age-matched, male spontaneously hypertensive rats used in these studies were direct descendants of the original strain developed by Okamoto & Aoki (1963). At the time of the experiment the animals were 20–24 weeks old. The blood pressures of the conscious animals were measured regularly from their twelfth week onwards at two weekly intervals by the indirect tail cuff method. At the end of the twentieth week the animals with systolic blood pressures of 170 mmHg and above were considered hypertensive (SH) and those with 110–130 mmHg were considered normotensive (NSH). Male rats of Wistar strain (NW) with blood pressure between 100–130 mmHg were used as control for comparison.

### *Perfused hindquarter preparation*

Animals were anaesthetized with a combination of sodium pentobarbitone (20 mg/kg, i.p.) and urethane (500 mg/kg, i.p.). The hindquarters of the rats were perfused at constant flow as described by Beck (1961). Blood was forced from the proximal to the distal part of the abdominal aorta by a peristaltic pump (DESAGA). Heparin 10 mg/kg was administered intravenously to all the animals, before cannulation of the aorta. The blood pressure and perfusion pressure

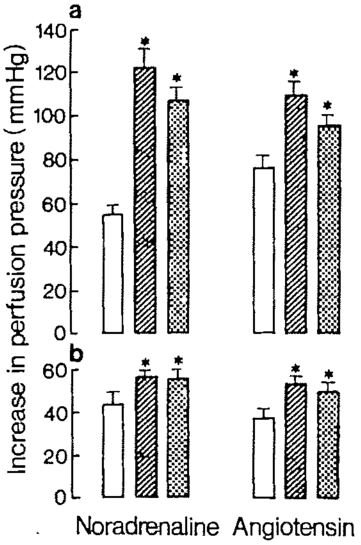

**Figure 1** Effect of noradrenaline and angiotensin II on (a) perfused mesenteric artery and (b) hindquarters preparations from age-matched normotensive Wistar (NW) rats (open columns) spontaneously hypertensive (SH) rats (cross-hatched columns) and normotensive rats from a spontaneously hypertensive colony (NSH) (dotted columns). The mean values are shown; vertical bars indicate s.e. mean. ($n = 10$ for each group).

* significantly different from control, $P < 0.01$ Student's *t* test).

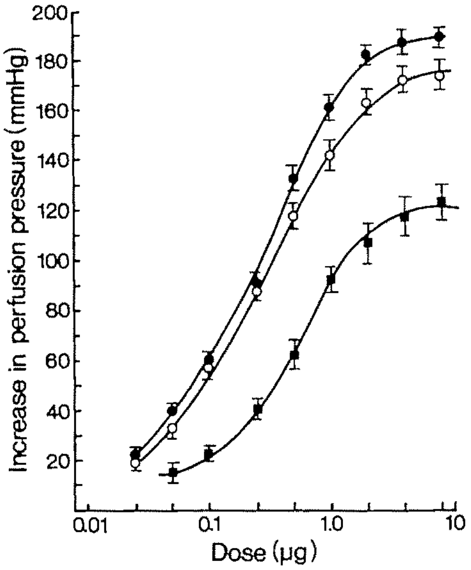

**Figure 2** Dose-response curves for the increase in perfusion pressure produced by noradrenaline (NA) in perfused mesenteric artery preparations from age matched (■) normotensive Wistar (NW) rats, (●) spontaneously hypertensive rats (SH) and (○) normotensive rats from a spontaneously hypertensive colony (NSH). The mean values are shown; vertical bars indicate s.e. mean. ($n = 8$ for each group).

were measured with Statham P23Db pressure transducers and recorded on a two-channel Hellige physiological recorder. The pump speed was initially set in such a way that the perfusion pressure was the same as the systemic blood pressure. Local intra-arterial injections into the hindquarters were given by needle puncture into the rubber tubing leading to the distal part of the aorta.

### *Perfused mesenteric artery preparation*

The general technique was similar to that described for the perfused hindquarters preparation. Blood from the carotid artery was forced by a peristaltic pump into the superior mesenteric artery. In addition, dose-response curves to NA were obtained by injection into the perfusion system. Injections were given at 5 min intervals and the maximum increase in perfusion pressure and 50% of these maximal responses ($M_{50}$) were calculated as described by Folkow *et al.* (1970b).

The results obtained were analysed statistically by Student's *t* test.

## Results

The body weights of the animals used were 176.7 ± 6.7 g for NW rats, 209.0 ± 4.9 g for SH rats and 206.7 ± 8 g for NSH rats. NSH and SH rats were significantly ($P < 0.01$) heavier than NW rats.

The mean blood pressures of anaesthetized NW, NSH and SH rats as measured in the carotid artery were 107 ± 4.2 mmHg, 117 ± 8.0 mmHg and 182 ± 5.2 mmHg respectively. Blood pressure of SH rats showed a significant ($P < 0.001$) difference compared to NW and NSH rats.

The vasoconstrictor responses to NA (0.5 µg) and angiotensin II (0.5 µg) were determined in the perfused hindquarters and mesenteric artery preparations of NW, NSH and SH rats. These agents, when given intra-arterially, elicited pressor responses in the perfusion pressure without any effect on the systemic blood pressure. The preparations obtained from NSH and SH rats exhibited significantly increased vasoconstrictor responses to NA and angiotensin II compared to NW rats. The responses of these agents in NSH rats did not differ significantly from those of SH rats. Vascular reactivity to NA and angiotensin II in the perfused mesenteric artery preparation was significantly greater than that in the perfused hindquarter preparation (Figure 1).

In addition, NA produced a dose-dependent vasoconstrictor response in the perfused mesenteric artery preparation. In preparations obtained from NSH and SH rats the dose-response curves produced by NA exhibited steeper slopes and higher maxima as compared with NW rats (Figure 2). The maximal pressor responses to NA in NSH rats (173.3 ± 5.9 mmHg) and SH rats (188.3 ± 4.2 mmHg) were found to be significantly ($P < 0.001$) increased as compared with NW rats (121.7 ± 7.2 mmHg). The $M_{50}$ values for NA in NSH rats (0.24 ± 0.01) and SH rats (0.25 ± 0.01) were also found to be significantly ($P < 0.001$) lower than those of NW rats (0.48 ± 0.024), while there was no significant difference between NSH and SH rats.

## Discussion

Previous studies in different types of experimental hypertensive animals have presented conflicting views concerning the relationship between increased vascular reactivity and the development of elevated blood pressure (Folkow *et al.*, 1958; Dupont & Sassard, 1974; Finch, 1975). Since hyper-reactivity can be demonstrated in both whole animal preparations and perfused arteries, the underlying mechanism for the increase in reactivity seems to be located in the blood vessels themselves. In the present study, the vascular reactivity to NA and angiotensin II was studied in perfused hindquarter and mesenteric artery preparations in different strains of rats of 5–6 months' age, since hypertension develops in SH rats at the age of 3–4 months.

The vascular reactivity to NA and angiotensin II was significantly increased in SH rats, compared to that in NW rats. However, it was surprising to find that NSH rats with lower blood pressure also exhibited significantly increased vascular reactivity compared to that of NW rats. The vascular reactivity to NA and angiotensin II has been found to be similar in SH and NSH rats. Finch (1975) has shown that lowering of blood pressure as a result of antihypertensive therapy failed to modify vascular reactivity to vasoconstrictor agents in the isolated perfused mesenteric preparation of DOCA/NaCl hypertensive rats. These results indicate that vascular reactivity to vasoconstrictor agents is not dependent on the blood pressure levels. This is in agreement with the work of Wayyes & Paterson (1975) in SH rats.

In addition it was observed that vascular reactivity in the perfused mesenteric artery preparation was significantly more elevated than that in the perfused hindquarters preparation. The different results obtained in these two preparations could probably be explained by the presence of intact blood vessels, including resistance vessels in the hindquarters but not in the mesenteric artery preparation.

Furthermore in the perfused mesenteric artery preparation from NSH and SH rats, the dose-response curves to NA exhibited steeper slopes and increased maximal responses as compared with NW rats. According to Folkow *et al.* (1970a) an increase of the wall/lumen ratio alters the shape of the dose-response curve for a vasoconstrictor agent.

These findings would suggest that adaptive/structural changes of the blood vessels could be expected not only in SH rats but also in NSH rats.

The results obtained from the present study raise some doubts about the role of increased vascular reactivity of the blood vessels in the development or maintenance of elevated blood pressure in SH rats. It appears that in addition to the hyper-responsiveness of blood vessels some other factor such as neurohumoral or chemical influences may also be responsible in elevating blood pressure in SH rats. Such a speculation can only be examined critically after other genetic changes in these animals have been identified because there is considerable evidence to indicate that hypertension in these animals is a polygenetic trait (Tanase, Suzuki, Ooshima, Yamori & Okamoto, 1972). Since alteration of adrenergic mechanism has been observed in SH rats, the role of central biogenic amines in the development of hypertension in SH rats needs clarification.

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*(Received April 20, 1976.*
*Revised July 20, 1976.)*

*Br. J. Pharmac.* (1977), 59, 247–252

# THE EFFECTS OF EDTA AND EGTA ON RENIN SECRETION

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1 The effects of the disodium salt of ethylenediamine tetra-acetate (EDTA) and 1,2,bis,2 amino-ethoxyethane-*NNN′N′*-tetra-acetic acid (EGTA) on renin secretion and vascular resistance were studied in the isolated perfused kidney of the rat.

2 Both substances produced a significant increase of renin release.

3 In the absence of calcium and magnesium, EDTA still increased renin release and there was now a considerable increase of perfusion pressure.

4 The rise of pressure but not the rise of renin was inhibited by the removal of potassium from the perfusate when EDTA was administered in the absence of calcium.

5 Propranolol and phenoxybenzamine had no effect on the vasoconstrictor action of EDTA.

6 EGTA was less effective as a renin releaser than EDTA until magnesium was removed from the perfusate. Further, it had only a small effect on perfusion pressure in contrast to EDTA.

## Introduction

The probable importance of calcium ion in the release of renin from the juxtaglomerular cells has been stressed in recent work from this laboratory. It was shown that lowering the external calcium concentration increased renin release and the inhibition of renin release produced by angiotensin was calcium-dependent (Vandongen & Peart, 1974a), and that lanthanum, which is known to block calcium flux across various cell membranes (Weiss, 1974), inhibits spontaneous renin release as well as that induced by isoprenaline and glucagon (Logan, Tenyi, Quesada, Peart, Breathnach & Martin, 1975). This latter inhibition was only overcome by the introduction of the disodium salt of ethylenediamine tetra-acetate (EDTA) and it was suggested that this might act by chelating and therefore removing lanthanum on the surface of the juxtaglomerular cells and then increasing calcium efflux. It therefore seemed important to study the actions of EDTA and 1,2,bis, 2-aminoethoxyethane-*NNN′N′*-tetra-acetic acid (EGTA), which have different affinities for calcium (Williams, 1972), on renin release, and at the same time to note their actions on renal vasoconstriction as a possible indicator of calcium flux since vasoconstriction is probably associated with the entry or increase of calcium within smooth muscle cells (Somlyo & Somlyo, 1968; Keatinge, 1972; Van Breemen, Farinas, Casteels, Gerba, Wuytack & Deth, 1973). The likely origin of juxtaglomerular cells from vascular smooth muscle cells (Barajas & Latta, 1967) makes this comparison especially apposite.

## Methods

### *Kidney perfusion*

Male Wistar rats (300–350 g) maintained on a normal diet, were anaesthetized with sodium pentobarbitone (0.1 mg/g) intraperitoneally and given 100 units heparin intravenously. The left kidney was isolated and perfused as described previously (Vandongen, Peart & Boyd, 1973). The perfusion fluid was usually 'Krebs-Ringer dextran' of the following composition (mmol/l): Na 135, Ca 3.7, K 6.0, Mg 1.2, glucose 10 and dextran 36 g/l (mol wt 70,000). This was equilibrated with 95% $O_2$ and 5% $CO_2$ at 37°C, and was delivered as pulsatile flow at a constant rate (usually 8 ml/min) by roller pump. Perfusion pressure was measured by transducer and Devices M2 recorder. The perfusion fluid was modified for different experiments and calcium and magnesium were omitted without ionic replacement. When potassium was omitted the composition of the fluid was (mmol/l): Na 135, Ca 2.5, choline chloride 5.5, Mg 1.0, glucose 10 and dextran 36 g/l (mol wt

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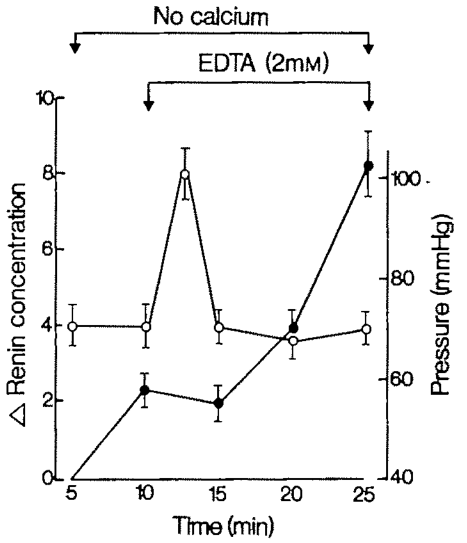

**Figure 1** The effect of EDTA in the absence of calcium on renin concentration (●) and renal perfusion pressure (O) in the isolated kidney of the rat.

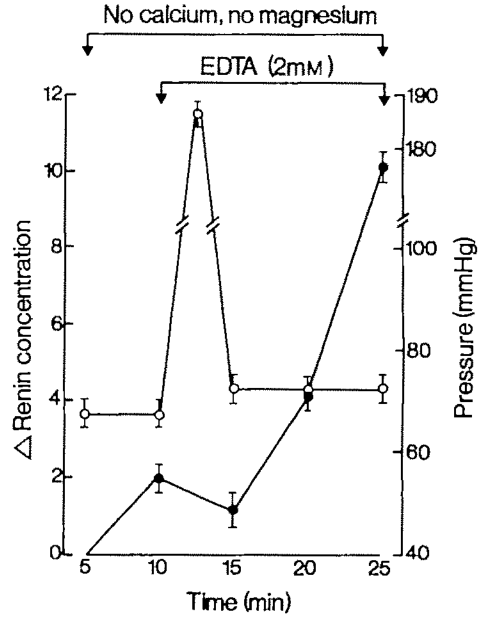

**Figure 2** The effect of EDTA in the absence of calcium and magnesium on renin concentration (●) and renal perfusion pressure (O) in the isolated kidney of the rat. Note the break in the scale for pressure due to the large rise.

70,000), buffered with Hepes (N-2-hydroxyethyl-piperazine-*N*′-2-ethane-sulphonic acid) (Sigma) to pH 7.4 and oxygenated with 100% $O_2$, and the control solution had KCl (K 6.0 mmol/l) in place of the choline chloride. Ethylenediamine tetra-acetate disodium salt (EDTA) and 1,2,bis,2 aminoethoxy-ethane-*NNN*′*N*′-tetra-acetic acid (EGTA) (Hopkin and Williams) were added to the perfusion fluids in various experiments in concentrations of 1 or 2 mmol/litre. The osmolarity of the buffers was checked by a freezing point depression method (Advanced Osmometer 3L; Advanced Instruments Inc., U.S.A.). Phenoxybenzamine (Smith Kline and French) and propranolol (ICI) dissolved in the appropriate perfusion fluid were infused separately into the arterial line through a needle in the tubing at 0.04 ml/minute.

### *Renin assay*

One ml from each perfusate sample (8 ml) was dialysed successively at pH 5 and pH 7.5 for 24 h at 4°C against phosphate buffers containing gentamicin (10 μg/ml) and EDTA to remove angiotensinases (Skinner, 1967). Samples were then incubated at 37°C with plasma from rats nephrectomized 24 h previously (1 ml perfusate with 0.4 ml plasma) which had been treated by similar dialysis procedures. The reaction was stopped by heating to 90°C for 10 minutes. The angiotensin I produced was measured by radioimmunoassay (Boyd, Adamson, Fitz & Peart, 1969), and the renin activity expressed as nmol of 5-isoleucine-angiotensin I generated $l^{-1}$ $h^{-1}$. The results were expressed as the ratio of the renin concentration at observed time to the renin concentration at zero time (△). All values given are means ± s.e. and significance was measured by Student's paired *t* test.

### *Types of infusion*

Initial experiments were carried out in which control infusions were made with the following solutions: Krebs ($n = 16$); calcium-free ($n = 5$); magnesium-free ($n = 5$); calcium and magnesium-free ($n = 5$); Hepes ($n = 5$). In successive experiments, after a control period of 10 min the perfusion fluid was changed to one containing either EDTA or EGTA and the effect on perfusion pressure and renin concentration measured during the next 15 minutes. In two other sets of experiments, the Krebs solution contained either propranolol (4 nmol/ml) ($n = 5$) or phenoxybenzamine (0.5 nmol/ml) ($n = 6$) throughout the control period and was continued when the EDTA was introduced.

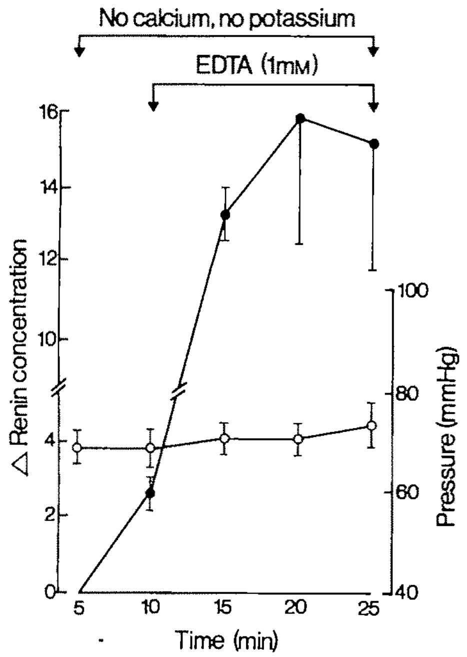

**Figure 3** The effect of EDTA in the absence of calcium and potassium on renin concentration (●) and renal perfusion pressure (O) in the isolated kidney of the rat.

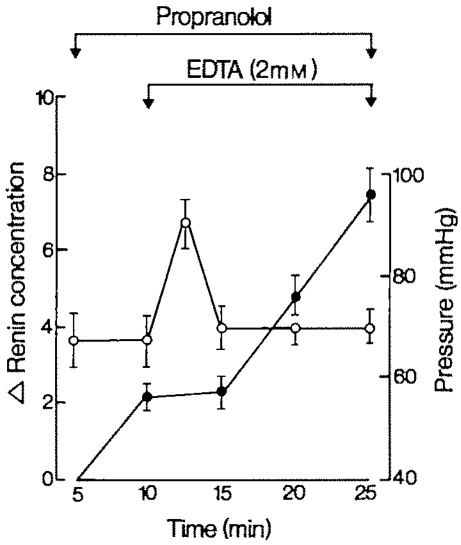

**Figure 4** The effect of propranolol (4 nmol/ml) on renin concentration (●) and renal perfusion pressure (O) in the isolated kidney of the rat.

**Results**

*Control infusions*

Comparison was made between the renin concentration in the sample collected after 5 min perfusion and after 10, 15, 20 and 25 min, and since the maximum difference was seen at 25 min, the result was expressed as the ratio of the concentrations at that time over the 5 min value. Krebs solution ($n=16$; $\Delta$ $2.1\pm0.2$); calcium-free ($n=5$; $\Delta$ $2.7\pm0.34$); magnesium-free ($n=5$; $\Delta$ $2.2\pm0.1$); calcium and magnesium-free ($n=5$; $\Delta$ $1.24\pm0.48$); Hepes solution ($n=5$; $\Delta$ $1.67\pm0.4$). Since the changes observed with EDTA and EGTA were very much greater, comparisons thereafter were not made with these controls but the differences within each experiment from the starting value were used. There were no changes of pressure with time in any of this group of experiments.

*EDTA with different solutions*

*Calcium free* ($n=8$) (Figure 1). The addition of EDTA (2 mmol/l) caused an immediate rise of pressure without a change in renin release followed by a gradual rise in renin over the next 10 min ($P<0.001$), while the pressure returned to its initial value within 5 minutes.

*Calcium and magnesium-free* ($n=5$) (Figure 2). The addition of EDTA (2 mmol/l) was associated with a very marked rise of pressure and a significant reduction in renin release ($P<0.05$). Continued perfusion led to a drop in pressure almost to the starting value within 5 min with a steady rise in renin secretion over 10 min ($P<0.001$).

*Calcium and potassium-free* ($n=4$) (Figure 3). The addition of EDTA did not cause a change of pressure in contrast to experiments where potassium was present (Figure 1) and renin release occurred promptly (within 5 min) (contrast Figure 1), and continued to rise over 10 min ($P<0.001$).

*Krebs solution with propranolol* ($n=5$) *or phenoxybenzamine* ($n=6$) (Figures 4 and 5). When EDTA (2 mmol/l) was introduced in the presence of either propranolol (4 nmol/ml) or phenoxybenzamine (0.5 nmol/ml), the immediate short-lived rise in pressure was unaffected and renin concentration rose significantly after the first 5 min as in the experiments in the absence of these drugs ($P<0.001$) (c.f. Figure 1).

*EGTA with different solutions*

*Calcium-free* ($n=5$) (Figure 6). EGTA (2 mmol/l) caused an immediate rise in renin secretion which was

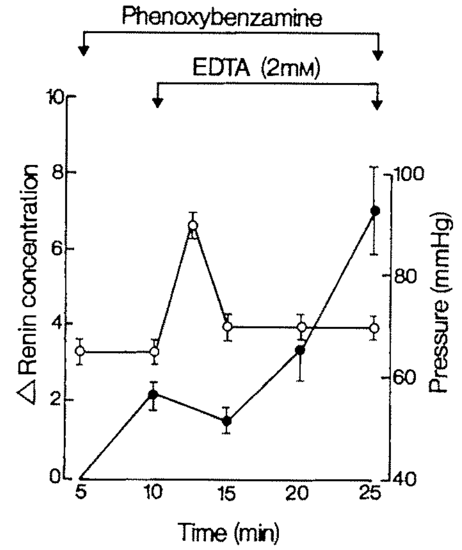

**Figure 5** The effect of phenoxybenzamine (0.5 nmol/ml) on renin concentration (●) and renal perfusion pressure (O) in the isolated kidney of the rat.

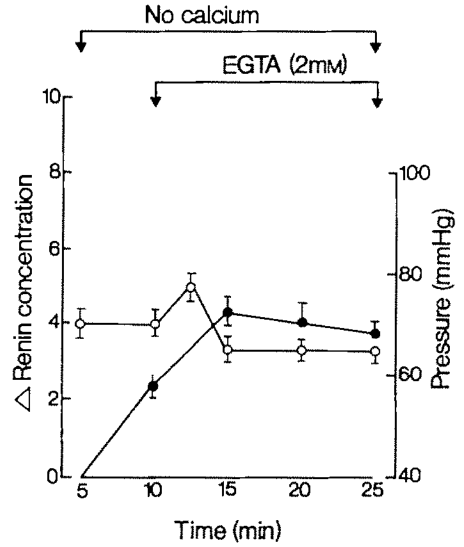

**Figure 6** The effect of EGTA in the absence of calcium on renin concentration (●) and renal perfusion pressure (O) in the isolated kidney of the rat.

not progressive after the first 5 min but remained significantly elevated ($P < 0.01$). The immediate rise of pressure was very slight ($P > 0.05$) and returned to a maintained level within 5 minutes.

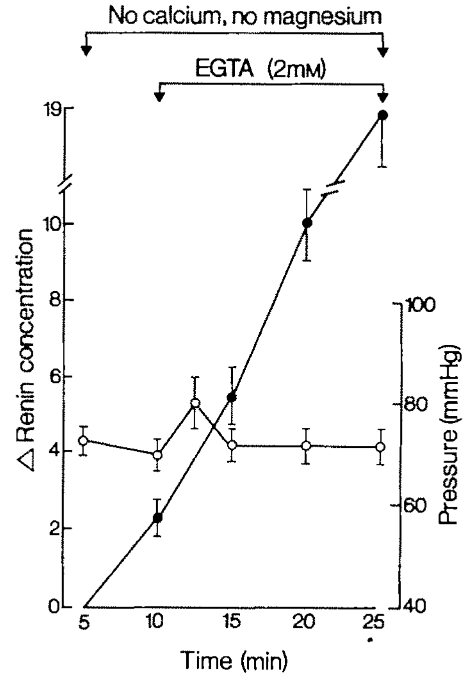

**Figure 7** The effect of EGTA in the absence of calcium and magnesium on renin concentration (●) and renal perfusion pressure (O) in the isolated kidney of the rat. Note the break in the renin concentration line because of the marked increase.

*Calcium and magnesium-free* ($n = 4$) (Figure 7). In contrast to the previous experiments, the introduction of EGTA (2 mmol/l) caused a rapid and increasing rise of renin secretion ($P < 0.001$). The pressure change was insignificant and transient ($P > 0.05$) and stayed on the baseline after 5 minutes.

## Discussion

In previous studies on the perfused kidney of the rat there was evidence that EDTA increased renin release (Vandongen & Peart, 1974a; Logan *et al.*, 1975; Logan, Tenyi, Peart, Breathnach & Martin, 1976). In the present series of experiments it is clear that EDTA in the absence of external calcium and magnesium caused a rise of renin release which was continued to the termination of the experiments. There was no significant difference between the experiments without calcium and without calcium and magnesium (Figures 1 and 2). The addition of propranolol or phenoxybenzamine when the perfusate contained both calcium and magnesium, did not prevent this steady rise in renin release due to EDTA (Figures 4 and 5). This therefore excludes the participation of $\alpha$- or $\beta$-adrenoceptor stimulation in this renin release. The effects of EDTA on perfusion pressure in this series of experiments is of great interest since the transient rise of pressure produced even in the absence of calcium (Figure 1) was greatly magnified in the absence of both calcium and magnesium (Figure 2) and this latter rise, which was confined to the first 5 min of EDTA perfusion, was associated with a significant fall in renin release. Can these findings be related to the hypothesis that renin inhibition is associated with an increased influx or rise of intracellular calcium ion (Vandongen & Peart, 1974b; Logan *et al.*, 1975; 1976)? It is conceivable that if EDTA caused a marked calcium gradient from the interior to the exterior of the cell, intracellular calcium stores would be released, leading to smooth muscle contraction, and in the juxtaglomerular cell to inhibition of renin release. As efflux increased there would be a subsequent fall of intracellular calcium, smooth muscle would relax and renin release would be increased. Certainly the contraction was not blocked by phenoxybenzamine and is presumably not mediated through $\alpha$-receptors. The effect of omitting potassium as well as calcium from the perfusate in the presence of EDTA (Figure 3) is of considerable interest since this abolished the rise of pressure, and the renin release was quicker and of the same eventual magnitude as in previous experiments (compare Figures 1 and 3). One possible explanation could be that external potassium is controlling calcium influx or release from the cell membrane and that in its absence efflux is favoured and renin release is therefore quicker (Figure 3). The cell membrane or intracellular processes within the smooth muscle and juxtaglomerular cell must be involved in the actions of EDTA and EGTA since purely removing calcium and magnesium from the perfusing medium with normal potassium content had a much smaller effect on renin release and none on pressure.

The effect of EGTA, which has a much greater affinity for calcium than for magnesium (Williams, 1972), therefore becomes of great importance and while it certainly stimulated renin release in the absence of calcium, it was not as active as EDTA (Figures 6 and 7) and the difference between renin levels at 25 min in the two series of experiments was significant ($P<0.005$). The omission of magnesium and calcium from the perfusate in the presence of EGTA then led to an immediate and continuous rise in renin release. The small pressor effect with EGTA in the absence of calcium had an inhibitory action on renin release. The small pressor effect with EGTA further indicates that there is unlikely to be any big intracellular shift of calcium ion in contrast to the results with EDTA (compare Figures 1 and 6). It is possible that an antagonism between external calcium and magnesium in relation to smooth muscle contraction is also revealed by the different effects of EDTA and EGTA (compare Figures 1, 2, 6 and 7). It is realized that interpretations of a complicated ionic interrelationship of this sort are dangerous and by concentrating on the possible importance of the flux and intracellular concentration of calcium ion, that other effects might be given too little emphasis. A rise of renin release might simply be due to increased cell membrane permeability caused by EDTA or EGTA without even the necessity for changes in ionic flux. Equally EDTA or EGTA may be active inside the cell and apart from manipulation of external ionic concentrations, there is no direct evidence presented here. The importance of magnesium in smooth muscle contraction has recently received more attention (Ford & Podolsky, 1970) and in other arteries of the rat, a relation between magnesium efflux and a ouabain-sensitive sodium pump has been postulated (Palatý, 1974). The vasoconstrictor effects of angiotensin which are certainly abolished in the rat kidney preparation by removal of calcium from the perfusate (Vandongen & Peart, 1974a), are increased by omitting magnesium in other preparations (Altura & Altura, 1971). Together with the present evidence, a relation between calcium, magnesium and potassium in respect of both smooth muscle contraction and renin release is strongly indicated by these postulated actions of EDTA and EGTA.

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*Br. J. Pharmac.* (1977), 59, 253–259

# EFFECT OF INDOMETHACIN AND RELATED DRUGS ON THE CALCIUM ION-DEPENDENT SECRETION OF LYSOSOMAL AND OTHER ENZYMES BY NEUTROPHIL POLYMORPHONUCLEAR LEUCOCYTES *In vitro*

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1 Rabbit isolated peritoneal neutrophil polymorphonuclear leucocytes were depleted of calcium by exposure for 1 h to calcium-free bathing fluid at 4°C.

2 Addition of calcium ions to the previously calcium-depleted cells during incubation at 37°C stimulated the release of $\beta$-glucuronidase and of lysozyme but not of lactate dehydrogenase.

3 Low concentrations of indomethacin, flufenamate or salicylate, such as those which occur in the blood plasma after therapeutic doses of these drugs, selectively inhibited the calcium-induced release of $\beta$-glucuronidase. The slight release of this enzyme which occurred in the absence of added calcium ions was not altered by these drugs, neither was the release of lactate dehydrogenase.

4 Release of lysozyme was inhibited by low concentrations of salicylate, amidopyrine or oxyphenbutazone, independent of the presence or absence of calcium ions.

5 Chloroquine, hydrocortisone or colchicine did not alter the release of leucocyte enzymes.

## Introduction

Indomethacin and related drugs inhibit many types of inflammation but the pharmacological basis of this effect is still obscure. One possibility is that these drugs inhibit the secretion of lysosomal enzymes by the neutrophil polymorphonuclear leucocytes (neutrophils) which attach themselves at an early stage of inflammation to the walls of the small blood vessels. Selective secretion by the neutrophils of their lysosomal contents occurs in response to a variety of physiological and pathological stimuli but in most cases stimulus-secretion coupling ceases under conditions of calcium depletion (Woodin & Wieneke, 1963; Henson, 1972; Becker & Showell, 1974; Goldstein, Horn, Kaplan & Weissmann, 1974; Ignarro, 1974b; Ignarro & George, 1974; Goldstein, Hoffstein & Weissmann, 1975; Smith & Ignarro, 1975). Indomethacin and related drugs deplete the membrane-bound stores of calcium in smooth muscle cells and vascular endothelial cells (Northover, B.J., 1971, 1972, 1973, 1975a, b; Northover, A.M., 1975). Thus, it was of interest to determine whether anti-inflammatory drugs would modify the calcium-dependent secretion of lysosomal and other enzymes by the neutrophils *in vitro*.

## Methods

Rabbits of the New Zealand White strain weighing 2 to 3 kg were injected intraperitoneally through a 0.8 mm external diameter stainless steel needle with 100 ml of sterile thioglycollate medium (United States Pharmacopoeia, 18th revision, 1970; supplied by Oxoid Ltd.). Between 17 and 22 h later each rabbit was lightly anaesthetized by inhalation of ether vapour and injected intraperitoneally with 250 ml of sterile 0.15 M sodium chloride solution containing heparin (4 iu/ml). After massaging the abdomen for 10 s the peritoneal fluid was withdrawn by gentle suction via a 1.5 mm external diameter plastic catheter the end of which was multiply perforated. The cells in the peritoneal fluid were sedimented at 22°C by centrifugation in 100 ml plastic tubes at 200 *g* for 4 minutes. The sedimented cells were washed twice by resuspension and recentrifugation at 200 *g* for 4 min at 4°C, each time using 100 ml of suspending medium having the following composition (mM): NaCl 150, KCl 3, glucose 10, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid 5, adjusted to pH 7.4 with NaOH. In some experiments 1.0 mM sodium 1,2-bis-2-aminoethoxyethane-*NNN'N'*-tetra-acetate (EGTA) was added to the suspending medium. When EGTA-containing medium was used as the washing fluid the cells were suspended in this fluid for the remainder of the experiment. Otherwise, the cells were washed with and suspended in EGTA-free medium throughout. The suspension of washed cells was adjusted to a total count of $5 \times 10^6$ cells/ml, of which 94 to 98% were neutrophils, the remainder being various types of mononuclear cells. Each rabbit served as a source of neutrophils on four occasions separated by intervals of a week or longer. Preliminary experiments showed that the proportion of neutrophils in the peritoneal washings decreased if animals were used more than four times or more frequently than once a week. The total time which elapsed between the withdrawal of the peritoneal fluid from a rabbit and the start of the incubation of the neutrophils as part of an experiment was 60 minutes.

Substances to be tested for their effect upon the release of enzymes from the neutrophils were added in a volume of 0.2 ml to 1.0 ml aliquots of leucocyte suspension in 10 mm diameter polypropylene tubes. The tubes were rotated at 30 rev/min in a rack inclined at 45° to the vertical in an incubator room at 37°C. After incubation for a pre-arranged time (40 min unless otherwise specified) the tubes were cooled in melting ice and then centrifuged at 3,000 *g* for 6 min at 4°C. Enzyme assays were performed in 1 cm cuvettes in a Unicam SP 500 spectrophotometer. In experiments where a drug was added to the leucocytes during incubation or the ionic composition of the suspending medium was altered, the drug or ion involved was added to all tubes, including the controls, during the subsequent enzyme assays. This precaution was necessary since some anti-inflammatory drugs have been reported to inhibit certain lysosomal enzymes such as $\beta$-glucuronidase and acid phosphatase (Anderson, 1968; 1970).

The results of all enzyme assays were expressed as a percentage of the activity of an aliquot of the supernatant obtained by centrifuging a sample of leucocyte suspension to which Triton X-100 (Rhome & Has, Philadelphia) had been added in a final concentration of 0.2%. It was assumed that Triton released the entire leucocytic content of enzymes into the suspending medium.

$\beta$-Glucuronidase activity was determined by pipetting a sample of the supernatant fluid into a glass test tube containing 1.5 ml of 0.2 M sodium acetate buffer at pH 4.5 and 0.2 ml of a 6 mM solution of phenolphthalein glucuronide (Sigma). The mixture was incubated at 56°C for 3 h after which the pH was adjusted to 11.0 by adding 2 ml of a 0.2 M solution of 2-amino-2-methyl-1-propanol, and the optical extinction measured at 550 nm. The red colour of the liberated phenolphthalein was used as a measure of the glucuronidase activity, as suggested by Fishman (1955).

Lysozyme activity was determined by pipetting a sample of the supernatant fluid into a glass test tube containing 2.5 ml of a freshly prepared suspension of 0.5 mg of freeze-dried *Micrococcus luteus* (Boehringer Mannheim) in 0.27 M sodium orthophosphate buffer at pH 6.0. The optical extinction at 510 nm was measured immediately after mixing and again after exactly 20 min incubation at 22°C. The reduction in optical extinction during this time was considered to be due to bacterial lysis and was used as a measure of lysozyme activity, as proposed by Smolelis & Hartsell (1949).

Lactate dehydrogenase activity was determined by reducing pyruvate with the reduced form of nicotinamide adenine dinucleotide. A sample of the supernatant fluid was pipetted into a glass test tube containing 50 μl of a 9 mM solution of the dinucleotide (BDH) and 3 ml of a 50 mM sodium orthophosphate buffer at pH 7.5 containing sodium pyruvate 0.3 mM. The optical extinction at 366 nm was measured immediately after mixing and again after exactly 5 min incubation at 22°C. The reduction in optical extinction during this time was taken as a measure of the dehydrogenase activity, as proposed by Wróblewski & La Due (1955).

Amidopyrine and colchicine were used as freshly prepared aqueous solutions. Chloroquine was dissolved in water in the form of its sulphate salt. Indomethacin, flufenamate, oxyphenbutazone, phenylacetate, gentisate and salicylate were dissolved in water as their respective sodium salts and the pH adjusted to 7.4. Hydrocortisone was dissolved in water as its sodium succinate derivative. The quoted concentrations of these drugs refer to their respective free acids, bases or alcohol.

## Results

### *Effect of calcium ions and of EGTA*

The release of enzymes from the neutrophils depended upon the duration of incubation at 37°C and upon the composition of the fluid in which the cells were suspended. Cells washed with and suspended in calcium-free suspending medium released very little of their enzyme content during incubation (Figure 1). Addition of calcium ions during incubation caused a time-dependent increase of the release of glucuronidase and lysozyme but not of lactate dehydrogenase (Figures 1 and 2). The effect of added calcium ions during incubation was more consistent when the leucocytes had been treated with EGTA than when they had not been, and hence EGTA-containing suspensions were employed for the remaining experiments. As would be expected, more added calcium was required in the presence of EGTA than in its absence to produce a 10% release of glucuronidase and lysozyme (Figure 2). Added calcium ions failed to modify the release of enzymes

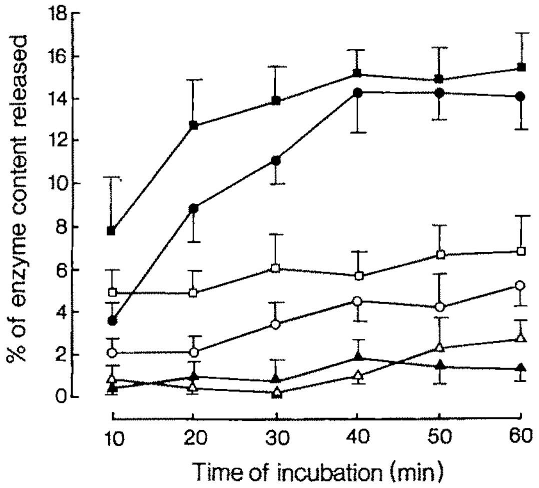

**Figure 1** Release of glucuronidase (○ and ●), lysozyme (□ and ■) and lactate dehydrogenase (△ and ▲) from neutrophils incubated for varying lengths of time in an EGTA-containing calcium-free suspending medium. Open symbols represent cells to which no calcium was added during incubation and closed symbols represent cells to which $CaCl_2$ (2 mM) was added. Each point is the mean of between 12 and 20 observations. Vertical bars represent one s.e.

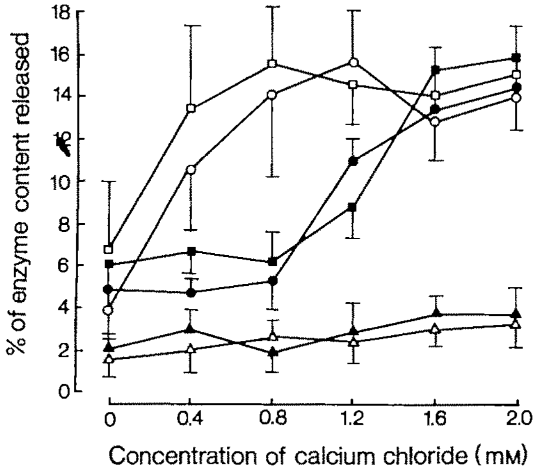

**Figure 2** Effect of calcium concentration on the release of glucuronidase (○ and ●), lysozyme (□ and ■) and lactate dehydrogenase (△ and ▲) from neutrophils incubated for 40 min in EGTA-free (open symbols) or in EGTA-containing (closed symbols) suspending medium. Each point is the mean of between 12 and 20 observations. Vertical bars represent one s.e.

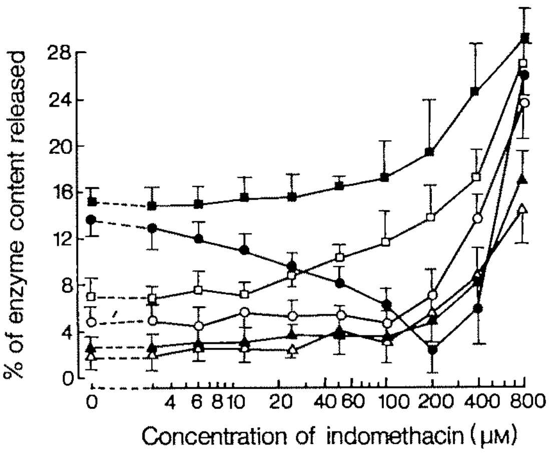

**Figure 3** Release of glucuronidase (○ and ●), lysozyme (□ and ■) and lactate dehydrogenase (△ and ▲) from neutrophils incubated for 40 min in EGTA-containing suspending medium to which various concentrations of indomethacin were added. Open symbols represent cells to which no calcium was added and closed symbols represent cells to which $CaCl_2$ (2 mM) was added during incubation. Each point is the mean of between 12 and 20 observations. Vertical bars represent one s.e.

when the neutrophils had been kept at 4°C for up to 1 hour.

### *Effect of indomethacin*

Addition of low concentrations of indomethacin to neutrophils produced a concentration-dependent inhibition of the calcium-induced release of glucuronidase (Figure 3). In contrast, the small release of glucuronidase observed in the absence of added calcium ions was not affected by low concentrations of the drug (Figure 3). The release of lysozyme both in the absence and in the presence of added calcium ions either was not altered or was slightly enhanced by low concentrations of indomethacin (Figure 3). Low concentrations of indomethacin did not alter the release of lactate dehydrogenase either in the presence or in the absence of added calcium. High concentrations of the drug caused a more or less equally enhanced release of all three enzymes, irrespective of the addition of calcium (Figure 3).

### *Effect of other anti-inflammatory drugs*

The effects of added flufenamate were similar to those of indomethacin (Table 1). In contrast, salicylate inhibited the release of both glucuronidase and lysozyme induced by the addition of calcium ions, although the release of lactate dehydrogenase was not altered by low concentrations of salicylate either in the presence or in the absence of added calcium ions, and higher concentrations of the drug caused a generalized enhancement of the release of all three enzymes (Table 1). In the absence of calcium, low concentrations of salicylate failed to alter the release of glucuronidase but did inhibit the release of lysozyme (Table 1). Both amidopyrine and oxyphenbutazone in low concentrations inhibited the release of lysozyme but failed to alter the release of glucuronidase or lactate dehydrogenase (Table 1). These actions of both drugs were independent of the presence of calcium ions. Chloroquine, hydrocortisone and colchicine each display anti-inflammatory activity *in vivo* under appropriate circumstances. Nevertheless, all three drugs failed to alter the release of leucocytic enzymes both in the presence and in the absence of calcium ions, although they were tested at concentrations at least as high as the plasma concentrations associated with anti-inflammatory effects *in vivo* (Table 1).

## Discussion

### *Effect of calcium ions on the release of enzymes*

The present investigation has shown that low concentrations of calcium ions, such as those which normally occur in the blood plasma, increase the release of both glucuronidase and lysozyme but not of lactate dehydrogenase from rabbit neutrophils previously depleted of calcium. Using human neutrophils, Goldstein *et al.* (1974) found that the addition of calcium ions substantially increased the release of lysozyme, whereas the release of glucuronidase was increased only slightly and lactate dehydrogenase release was unaltered.

### *Effects of indomethacin, flufenamate and salicylate on the release of enzymes*

In the present experiments low concentrations of indomethacin, flufenamate and salicylate inhibited the calcium-induced release of glucuronidase in a

**Table 1** Effect of drugs on the release of enzymes from neutrophils incubated in EGTA-containing suspending medium with and without added calcium chloride

| | | *Percentage release of enzymes** | | | | | |
|---|---|---|---|---|---|---|---|
| | *Concentration* | *β-Glucuronidase* | | *Lysozyme* | | *Lactate dehydrogenase* | |
| *Drug* | *(M)* | *Without calcium* | *With calcium†* | *Without calcium* | *With calcium* | *Without calcium* | *With calcium* |
| Control | — | 5 ± 1 | 14 ± 2 | 7 ± 2 | 15 ± 2 | 2 ± 1 | 2 ± 1 |
| Flufenamate | $4 \times 10^{-6}$ | 5 ± 2 | 13 ± 2 | 6 ± 1 | 15 ± 2 | 3 ± 1 | 3 ± 1 |
| Flufenamate | $8 \times 10^{-6}$ | 4 ± 1 | 10 ± 1‡ | 7 ± 2 | 14 ± 2 | 2 ± 1 | 1 ± 1 |
| Flufenamate | $1.5 \times 10^{-5}$ | 4 ± 1 | 8 ± 2‡ | 8 ± 2 | 16 ± 2 | 2 ± 2 | 2 ± 1 |
| Flufenamate | $3 \times 10^{-5}$ | 6 ± 2 | 5 ± 1‡ | 7 ± 2 | 15 ± 3 | 3 ± 1 | 3 ± 2 |
| Flufenamate | $6 \times 10^{-5}$ | 5 ± 2 | 2 ± 1‡ | 9 ± 2 | 18 ± 2 | 3 ± 1 | 4 ± 2 |
| Flufenamate | $1.2 \times 10^{-4}$ | 17 ± 2‡ | 10 ± 2 | 11 ± 3 | 29 ± 2‡ | 8 ± 2‡ | 12 ± 3‡ |
| Flufenamate | $2.4 \times 10^{-4}$ | 20 ± 2‡ | 23 ± 2‡ | 36 ± 4‡ | 34 ± 3‡ | 14 ± 2‡ | 28 ± 3‡ |
| Salicylate | $1.2 \times 10^{-3}$ | 5 ± 1 | 12 ± 2 | 6 ± 1 | 10 ± 2 | 2 ± 1 | 3 ± 1 |
| Salicylate | $2.5 \times 10^{-3}$ | 5 ± 2 | 6 ± 1‡ | 3 ± 1‡ | 8 ± 1‡ | 3 ± 1 | 3 ± 1 |
| Salicylate | $5 \times 10^{-3}$ | 6 ± 1 | 5 ± 1‡ | 4 ± 1‡ | 11 ± 2 | 2 ± 1 | 6 ± 2‡ |
| Salicylate | $1 \times 10^{-2}$ | 12 ± 2‡ | 23 ± 2‡ | 17 ± 2‡ | 28 ± 3‡ | 10 ± 2‡ | 22 ± 3‡ |
| Amidopyrine | $5 \times 10^{-4}$ | 6 ± 1 | 13 ± 3 | 4 ± 1 | 9 ± 2 | 3 ± 1 | 2 ± 1 |
| Amidopyrine | $1 \times 10^{-3}$ | 7 ± 1 | 16 ± 2 | 3 ± 1‡ | 7 ± 2‡ | 3 ± 2 | 1 ± 1 |
| Amidopyrine | $2 \times 10^{-3}$ | 19 ± 2‡ | 25 ± 2‡ | 20 ± 3‡ | 27 ± 3‡ | 15 ± 2‡ | 11 ± 2‡ |
| Oxyphenbutazone | $3 \times 10^{-4}$ | 4 ± 1 | 15 ± 2 | 6 ± 1 | 11 ± 1 | 3 ± 1 | 1 ± 1 |
| Oxyphenbutazone | $6 \times 10^{-4}$ | 7 ± 1 | 16 ± 2 | 2 ± 1‡ | 5 ± 1‡ | 2 ± 1 | 4 ± 1 |
| Oxyphenbutazone | $1.2 \times 10^{-3}$ | 23 ± 3‡ | 17 ± 2 | 16 ± 2‡ | 20 ± 2‡ | 10 ± 2‡ | 13 ± 2‡ |
| Chloroquine | $2 \times 10^{-5}$ | 6 ± 1 | 17 ± 2 | 10 ± 2 | 19 ± 2 | 3 ± 1 | 5 ± 2 |
| Colchicine | $2.4 \times 10^{-7}$ | 3 ± 1 | 12 ± 1 | 5 ± 1 | 13 ± 2 | 1 ± 1 | 2 ± 1 |
| Colchicine | $2.4 \times 10^{-6}$ | 7 ± 1 | 16 ± 2 | 9 ± 1 | 18 ± 2 | 2 ± 1 | 4 ± 1 |
| Hydrocortisone | $3 \times 10^{-4}$ | 5 ± 1 | 13 ± 1 | 8 ± 2 | 14 ± 2 | 2 ± 1 | 3 ± 1 |
| Phenylacetate | $1 \times 10^{-2}$ | 4 ± 1 | 16 ± 2 | 3 ± 1‡ | 6 ± 1‡ | 3 ± 1 | 2 ± 1 |
| Gentisate | $1 \times 10^{-2}$ | 4 ± 1 | 18 ± 2 | 2 ± 1‡ | 5 ± 1‡ | 4 ± 1 | 1 ± 1 |

* The mean of at least 12 observations. † The concentration of calcium added was 2 mM.
Values marked ‡ are significantly different (Student's *t* test, $P < 0.05$) from their corresponding control values.

concentration-dependent manner. In the case of indomethacin and flufenamate the inhibition disappeared in the absence of calcium ions in the suspending medium. The release of lactate dehydrogenase was unaffected by low concentrations of all three drugs. Drug concentrations which selectively inhibited the release of glucuronidase are comparable to those which are attained in the blood plasma in therapeutic use (Hucker, Zacchei, Cox, Brodie & Cantwell, 1966; Rothermich, 1966; Paulus, Siegel, Mongan, Okun & Calabro, 1971; Huidberg, Lausen & Jansen, 1972). On the other hand, the higher concentrations required to produce an unselective release of all three enzymes are probably not attained in the blood plasma after therapeutic doses.

Previous workers have shown that indomethacin, salicylate and some pharmacologically related drugs inhibit the release of glucuronidase from human neutrophils exposed either to phagocytosable or to opsonized solid materials (Andrews & Phelps, 1971; Perper & Oronsky, 1974). The present work indicates that the release of glucuronidase from rabbit neutrophils in response to a soluble stimulus may also be inhibited by these drugs. Nevertheless, at least two previous groups of workers have failed to show any inhibition of lysosomal enzyme release from phagocytosing neutrophils after treatment with salicylate in the concentration range used here (Wright & Malawista, 1973; Hawkins, 1974). Stimulus-secretion coupling mechanisms probably vary with different types of stimuli and may not be inhibited equally by drugs.

In general, the present findings lend support to the suggestion of Perper & Oronsky (1974) that part of the anti-inflammatory action of indomethacin, flufenamate and salicylate may be due to inhibition of the release of lysosomal enzymes from neutrophils in inflamed tissues. This is made more probable by the fact that phenylacetate and gentisate, non-anti-inflammatory analogues of indomethacin and salicylate respectively (Northover, 1963; 1964; Durant, Smith, Spickett & Szarvasi, 1965) both failed to alter the release of glucuronidase in the present experiments (Table 1).

### *Effects of other anti-inflammatory drugs on the release of enzymes*

In concentrations comparable with those occurring in the blood plasma during effective anti-inflammatory therapy, no inhibition of glucuronidase release was found in the present experiments with hydrocortisone, colchicine, chloroquine, amidopyrine or oxyphenbutazone. Previous workers, using phagocytosable or opsonized solid materials as the stimulus for release, have demonstrated an inhibition of lysosomal enzyme release from neutrophils with many of these drugs (Rajan, 1966; Andrews & Phelps, 1971; Oronsky, Ignarro & Perper, 1973; Weissmann, 1973; Wright & Malawista, 1973; Hawkins, 1974; Ignarro, 1974a; Perper & Oronsky, 1974; Ackerman & Beebe, 1975; Goldstein, 1975; Lewis & Day, 1975; Malawista, 1975; Oronsky & Perper, 1975). As noted previously, stimulus-secretion coupling mechanisms may vary with the nature of the stimulus involved, and consequently may vary in their capability of being inhibited by drugs. Perhaps the only anti-inflammatory drugs which inhibit the release of glucuronidase under the conditions of the present experiments are those that prevent the entry of calcium ions into the leucocyte.

### *Intra-leucocytic distribution of enzymes*

Rabbit neutrophils possess at least two morphologically distinct types of enzyme-containing structures derived from the Golgi apparatus, known as the azurophil and the specific granules respectively (Bainton & Farquhar, 1966). The azurophil granules store almost all of the glucuronidase whilst the specific granules contain a majority of the lysozyme (Baggiolini, Hirsch & DeDuve, 1969; 1970). The azurophil granules are typical of lysosomes in other types of cells and possess a full complement of acid hydrolases (Bainton & Farquhar, 1968a, b; Dewald, Rindler-Ludwig, Betz & Baggiolini, 1975). The specific granules are not typical lysosomes in their enzymatic content and their physiological and pathological significance is uncertain. In contrast to these two granule-bound enzymes, lactate dehydrogenase is a soluble cytoplasmic enzyme. Douwes (1973) has reported that indomethacin and some related drugs promote the release of lysozyme from phagocytosing neutrophils but inhibit the release of acid phosphatase, a typical lysosomal enzyme. A rather similar disparity between the effect of indomethacin on the release of glucuronidase and lysozyme was demonstrated in the present work. Since the majority of the lysozyme of the rabbit neutrophil is stored in the non-lysosomal specific granules this is readily understandable.

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*(Received May 27, 1976.*
*Revised August 11, 1976.)*

*Br. J. Pharmac.* (1977), 59, 261–268

# THE ROLE OF BACTERIAL CONTAMINATION IN THE ISOLATION OF APPARENT ANTI-INFLAMMATORY FACTORS FROM RABBIT ANTI-LYMPHOCYTIC SERUM

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1 Rabbit anti-guinea-pig lymphocytic serum was fractionated by gel filtration to obtain partially purified materials possessing anti-inflammatory activity. The pharmacological properties of these materials were then studied.

2 Two fractions were found which reproducibly contained significant activity. One of these activities caused inflammation at the site of injection and was associated with high molecular weight protein (200,000). The other activity was found in a low molecular weight fraction but was shown to be due to small amounts of endotoxin from Gram negative bacteria. These organisms contaminated the fractions in spite of the recommended precautions for gel filtration having been taken.

3 The endotoxin-containing fraction completely abolished leucocyte infiltration into the rat foot which had been injected with kaolin. It had no apparent effect on circulating haemolytic complement. It caused maximal elevation of serum 11-hydroxycorticosteroid concentrations and was found to cause the release of pharmacologically active amines. Many of the previously reported naturally occurring anti-inflammatory substances have similar pharmacological properties to those of the endotoxin-containing fraction.

4 It was concluded that doubt will exist about the presence of anti-inflammatory factors in mammalian body fluids unless stringent precautions are taken to exclude measurable bacterial contamination.

5 These experiments also cast doubt on the validity of accepted procedures for excluding microbial growth from columns used in the fractionation of serum.

## Introduction

It has been known for many years that inflammation at one site in an animal is reduced when an inflammatory response has been previously elicited at another site (Laden, Quentin Blackwell & Fosdick, 1958). Several hypotheses have been put forward to explain this phenomenon including the postulated existence in the blood of substances with anti-inflammatory activity.

Reports have appeared in the literature describing the occurrence of anti-inflammatory activity in exudates from granuloma pouches (DiPasquale, Girerd, Beach & Steinetz, 1963; Billingham, Robinson & Robson, 1969) and in serum from rats injured by laparotomy (van Gool, Schreuder & Ladiges, 1974), in rabbit anti-lymphocytic serum (Billingham, Robinson & Gaugas, 1970) and in normal human serum (McArthur, Smith & Freeman, 1972).

In this paper we have searched for such active substances in rabbit anti-lymphocytic serum which had been fractionated under conditions where some bacterial growth occurred in spite of standard precautions and also under conditions where bacterial contamination was reduced by stringent methods.

We have examined some of the pharmacological properties of our fractions to ascertain if they are similar to those of active fractions described by other workers.

## Methods

### *Preparation of anti-lymphocytic serum*

Rabbit anti-guinea-pig lymphocytic serum was raised as described by Billingham, *et al.* (1970). New Zealand White rabbits (Ranch Rabbits, Copthorne, Sussex) under pentobarbitone sodium (Abbott) anaesthesia, were bled from the carotid artery through a polythene cannula into 100 ml polypropylene centrifuge tubes seven days after the final injection of guinea-pig thymocytes. The blood was allowed to clot for at least 2 h at room temperature or occasionally overnight at 4°C before centrifugation at 2000 *g* for 20 minutes. The serum was freeze dried and stored at −20°C until required. It was not sterile.

Sterile anti-lymphocytic serum was prepared in a similar manner except that the rabbits were bled under aseptic conditions. The dissection area was treated with 0·5% Hibitane (ICI) in 70% ethanol. The cannulae were sterilized in a partially evacuated dessicator containing formaldehyde vapour for 16 h at room temperature and the blood was collected in autoclaved blood bottles in which clotting was permitted to take place as before. The blood was centrifuged and the serum siphoned into autoclaved bottles before freeze drying in a formaldehyde sterilized freeze drier. The serum was stored as before. No bacteria could be cultured from it.

### *Fractionation of serum*

The fractions were separated by gel filtration on columns of Sephadex G-150 or G-25 (medium grade, Pharmacia), equilibrated with 20 mM $NaH_2PO_4$, 200 mM NaCl, adjusted to pH 6.0 with NaOH. Experimental details of typical separations are given in the legends to Figures 1 and 2.

For separation on Sephadex G-25, the sample was applied by upward flow. The column was then inverted and elution continued by downward flow. By this inversion technique a sharper application band of the very viscous protein mixture was achieved. The column eluates were monitored at 254 nm (LKB Uvicord 1) and by electrical conductivity to detect changes in salt concentration (Radiometer CDM 2e conductivity meter equipped with a flow cell). The columns were kept flowing when not in use and were occasionally flushed with buffer containing 0.02% sodium azide, as recommended by Pharmacia (1970), in order to keep bacterial growth to a minimum. The fractions were pooled as indicated in Figures 1 and 2 and dialysed four times against at least 10 vol of deionized water, before freeze drying and subsequent testing. Columns were sterilized for some experiments by autoclaving the Sephadex, buffers and all glassware for 20 min at 121°C and leaving the plastic parts of the columns in contact with 1.0% Hycolin (William Pearson Ltd.) for 16 hours. Fractions from the sterilized columns were dialysed at 4°C to keep microbial growth to a minimum.

### *Measurement of anti-inflammatory activity*

Anti-inflammatory activity was assessed by the rat hind paw kaolin oedema test. Groups of six male Wistar rats (Anglia Laboratory Animals) weighing 180–200 g were injected with the test sample in either 1 ml of 0.9% w/v NaCl solution (saline) given subcutaneously into the adductor region or in 0.1 ml saline into the plantar region of the left hind limb. Control animals received the same volume of saline alone. Kaolin (25%, Hopkins & Williams) in 0.05 ml saline was injected 30 min later into the plantar region of the right hind paw. The volume of each paw was measured plethysmographically before and at intervals during the inflammatory response. By this method both the inflammatory and anti-inflammatory responses of the fractions could be assessed simultaneously in the same animal.

Anti-inflammatory activity was compared to that of indomethacin (Merck, Sharp & Dohme) standards injected similarly and is expressed as a potency relative to indomethacin (mg indomethacin equipotent to 1 mg freeze dried sample). Indomethacin (1.75–15 mg) was dissolved in 95% ethanol (0.5 ml), neutralized with 1M NaOH and diluted with saline to a final volume of 7 ml; 1 ml was injected subcutaneously in each rat.

### *Preparation of rat paws for histology*

Histological examination of the rat paws was carried out 6 h after kaolin injection. The animals were killed with chloroform vapour, the paws were excised, cut longitudinally into two parts and fixed in 10% buffered formalin pH 7.0 for a minimum of 24 hours. The paws were decalcified in 15% disodium edetate (EDTA) pH 7.0 and the end point (3–4 weeks) was determined by X-ray of the specimen. They were then dehydrated through an ascending series of alcohols, cleared in xylene and embedded in Fibrowax (m.p. 56°C; Raymond A. Lamb). Sections (5 μm) were cut on a Leitz base sledge microtome and stained in Ehrlich's haematoxylin and eosin.

### *Measurement of serum concentrations of corticosteroids and complement*

Rat blood samples were obtained, under pentobarbitone anaesthesia, by cannulation of the dorsal aorta with a Venocut winged infusion set (Argyle). The serum was prepared as for rabbit serum.

Total serum haemolytic complement was measured by the method of Kabat & Mayer (1961).

Serum 11-hydroxysteroid concentrations were determined by a modification of the method of Lowry, McMartin & Peters (1973). The steroids were extracted from the serum (2 ml) by gentle shaking with petroleum ether (boiling range 60–80°C, 6 ml) for approx. 20 minutes. After centrifugation the top layer was discarded and the lower layer (1 ml) was extracted with deionized water (2 ml) and dichloromethane (4 ml) by gentle shaking for 40 minutes. After centrifuging the lower dichloromethane layer (1 ml) was assayed for steroids by the above method.

### *Depletion of pharmacologically active amines*

Rats were depleted of 5-hydroxytryptamine and histamine by injection (i.p.) of 0.12 ml per rat of 1 mg/ml compound 48/80 (Burroughs Wellcome) twice daily for 3 days and 0.24 ml of 1 mg/ml 48/80 twice on the fourth day. The rats were used 5 h after the final injection.

### *Identification and culture of bacteria*

Bacteria were cultured initially in Trypticase Soy Broth (B.B.L.) incubated for approximately 18 h at 37°C or 22°C. Gram stains (Jarvis, 1973) were performed on the cultures. All secondary cultures from the broth were subcultured separately on Trypticase Soy Agar, MacConkey Agar, Cystine Lactose Electrolyte Deficient Agar and Pseudosel Agar (B.B.L.) incubated at 37°C for approximately 18 hours. Five morphologically similar colonies were subjected to standard biochemical tests and subsequently identified (Cowan, 1974). Each culture was concurrently checked for purity by culturing it on Trypticase Soy Agar.

All identified organisms were cultured in Trypticase Soy Broth at their optimum temperature for 18 h and washed three times with 0.1% peptone water by centrifugation at 6000 *g* for 5 min, once with deionized water and then freeze dried ready for testing for anti-inflammatory activity.

Bacterial endotoxin was detected by the *Limulus* amoebocyte lysate gelation test of Levin, Poore, Zauber & Oser (1970) as described in Sigma Technical Bulletin No. 210 (1973). The commercial endotoxin was *Escherichia coli* lipopolysaccharide type 1 from serotype 0127:88 (Sigma).

## Results

### *Separation of anti-inflammatory fractions*

When non-sterile anti-lymphocytic serum (specific activity, s.c., 0.004 mg indomethacin equivalents per mg) was chromatographed on a column of Sephadex G-150, two peaks were shown to be associated with anti-inflammatory activity after dialysis and freeze drying of the eluate (Figure 1, Table 1). This procedure took approximately three days and was carried out at room temperature. The anti-inflammatory activity (Table 1) in pool 2 eluted from the column with an apparent molecular weight of about 200,000 and had an activity (s.c.) of 0.01 mg indomethacin equivalents/mg in the kaolin paw oedema test. The active material in pool 5 eluted from the column with the serum salts (conductivity peak) and consequently had an apparent molecular weight of less than 5000. It was found to have an activity (s.c. of 0.08 mg indomethacin equivalents per mg.

In order to fractionate the low molecular weight components with the higher specific activity more quickly and with better resolution, anti-lymphocytic serum was applied to a column of medium grade Sephadex G-25 (Figure 2). Two active fractions were again eluted (Table 1). The first eluted with all the serum proteins which are totally excluded by this gel. This corresponded to the expected behaviour of the previously observed fraction with low specific activity, having a molecular weight of 200,000. A second peak of activity eluted with an apparent molecular weight of 1400 and had a specific activity (s.c.) of 0.44 mg indomethacin equivalents per mg (Table 1). Pool 2 from the Sephadex G-150 column and the low molecular weight active fraction from the Sephadex G-25 column (pool 3) were dialysed separately against

**Table 1** Typical specific anti-inflammatory activities of pools from sterile and non-sterile Sephadex G-150 and G-25 columns

| | *Sephadex G-150* | | | | | *Sephadex G-25* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Non-sterile* | | *Sterile* | | | *Non-sterile* | | *Sterile* | |
| *Pool No.* | *Activity/mg* | *mg/kg* | *Activity/mg* | *mg/kg* | *Pool No.* | *Activity/mg* | *mg/kg* | *Activity/mg* | *mg/kg* |
| 1 | NS | 200 | NS | 40 | 1 | 0.01* | 315 | 0.005* | 350 |
| 2 | 0.01* | 200 | 0.02* | 160 | 2 | NS | 10 | NS | 15 |
| 3 | NS | 200 | NS | 220 | 3 | 0.44* | 9 | NS | 15 |
| 4 | NS | 200 | | | 4 | NS | 10 | NS | 15 |
| 5 | 0.08* | 50 | NS | 42 | | | | | |

Pools were obtained from the columns as described in Figures 1 and 2. These were assayed in the rat paw kaolin oedema test. Their specific activities by subcutaneous injection relative to indomethacin, were calculated as described in the Methods section. Units are mg indomethacin equivalent to 1 mg freeze dried sample.

* Values significantly different ($P < 0.05$) from the saline controls. NS = not significantly different from controls at the dose used.

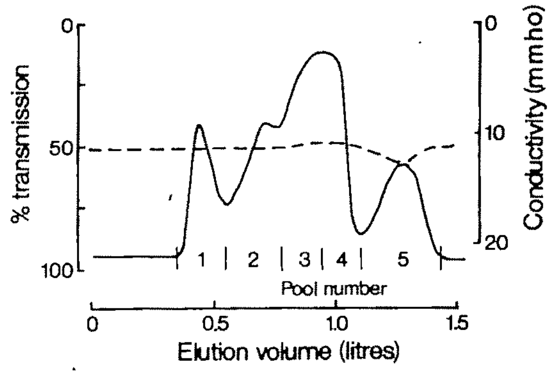

**Figure 1** A typical fractionation of rabbit anti-guinea-pig lymphocytic serum on Sephadex G-150. Freeze dried serum (3 g) was dissolved in 20 mM $NaH_2PO_4$, 200 mM NaCl adjusted to pH 6.0 with NaOH (25 ml) and applied to a 4.8 x 85 cm column of Sephadex G-150 eluted by upward flow at approximately 60 ml/hour. The eluate was monitored at 254 nm (—) and by electrical conductivity (– – –). The fractions were pooled as indicated.

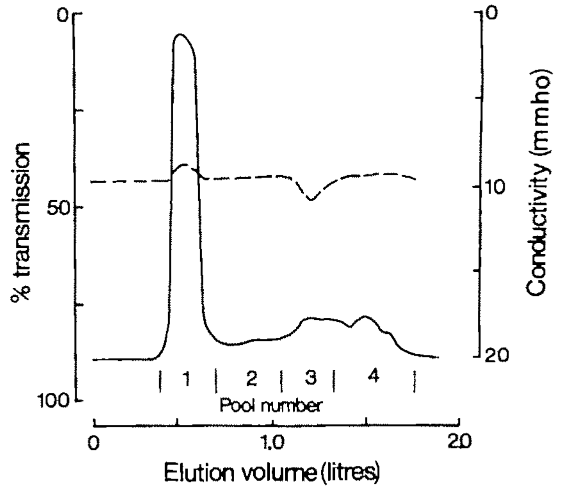

**Figure 2** A typical fractionation of rabbit anti-guinea-pig lymphocytic serum on Sephadex G-25. Freeze dried serum (10 g) was dissolved in 20 mM $NaH_2PO_4$, 200 mM NaCl adjusted to pH 6.0 with NaOH (60 ml) and applied to the 5.0 x 80 cm column of medium grade Sephadex G-25. The eluate was monitored at 254 nm (—) and by electrical conductivity (– – –). Fractions were pooled as indicated.

deionized water, freeze dried and used for further studies. The high molecular weight component from Sephadex G-150 chromatography will be referred to as fraction A and the apparently low molecular weight fraction from Sephadex G-25 chromatography as fraction B.

Fraction B did not dialyse through Visking tubing and was rechromatographed on a similar Sephadex G-25 column to ascertain whether any change had taken place. Its elution behaviour was then that of a material with a high molecular weight (greater than 5000). It was subsequently found that this active material was excluded by Sepharose 6B gel which indicates a molecular weight of greater than $4 \times 10^6$. This apparent change in molecular weight was investigated by taking samples of the fraction B from several columns, culturing and Gram staining them. Examination under the microscope showed the presence of Gram negative bacteria which, when subcultured, were identified as *Pseudomonas aeruginosa, Escherichia coli,* occasional *Enterobacter agglomerans* and an unidentified lactose-fermenting rod.

In order to assess the effects due to these bacteria, sterile rabbit anti-lymphocytic serum (activity 0.004 mg indomethacin equivalents per mg, s.c.) was chromatographed on an autoclaved column of Sephadex G-25. A similar fractionation to that shown in Figure 2 was achieved and no Gram negative bacteria could be cultured from the dialysed fractions. After freeze-drying in a sterile freeze-drier, the fractions were tested for anti-inflammatory activity. The excluded peak was found to contain low activity (0.005 mg indomethacin equivalents per mg, s.c.), but the low molecular weight fraction now contained no detectable activity (Table 1).

The same result was obtained when sterile anti-lymphocytic serum was chromatographed similarly on an autoclaved column of Sephadex G-150. The active fraction with a molecular weight of 200,000 (fraction A) was obtained and the low molecular weight fraction was inactive.

### *Anti-inflammatory activity of contaminating bacteria*

Each species of contaminating organism was cultured in fluid medium, collected and washed. The freeze dried cells were tested for anti-inflammatory activity (s.c.) (Table 2). It was found that *E. coli* was highly active, *Ent. agglomerans* was less active and *Ps. aeruginosa* and the lactose-fermenting bacterium had no detectable activity. A commercial preparation of *E. coli* endotoxin was also tested for anti-inflammatory activity and found to be 120 times as active as indomethacin (w/w).

A number of samples of fraction B were, therefore, prepared by both non-sterile and sterile procedures. These were tested for both anti-inflammatory activity and for endotoxin content by the *Limulus* amoebocyte lysate gelation test (Table 3).

Since some fractions from the columns were found

**Table 2** Specific anti-inflammatory activity of the freeze dried, washed bacterial cells cultured from the column fractions.

| *Species* | *Activity/mg* | *mg/kg* |
|---|---|---|
| *Escherichia coli* | 7.24* | 0.5 |
| *Pseudomonas aeruginosa* | NS | 2.5 |
| Unidentified lactose fermenter | NS | 0.5 |
| *Enterobacter agglomerans* | 1.26* | 2.5 |

Experimental details and units are as described in Table 1.

to contain anti-inflammatory activity and others not, the growth of bacteria was measured in each pool after sterile anti-lymphocytic serum was chromatographed on a column of Sephadex G-25 as before. The fractions were pooled in the manner exemplified by Figure 2, and 75 ml portions of each were innoculated with the arbitrary amount of $10^5$ cells *E. coli* in 0.1% peptone (1 ml). Growth was allowed to take place at room temperature and the amount of contamination was assessed by counting viable cells and measuring the endotoxin content after 3 days. Table 4 shows that the highest concentration of both viable cells and endotoxin was found in pool 3 which corresponds to the fraction B.

*Pharmacological effects of fractions A and B*

Fraction A, prepared as in Figure 1 was found to cause a severe, prolonged local inflammation when injected into the rat food pad (Figure 3). At the dose used only a small anti-inflammatory effect was observed.

Fraction B, prepared as in Figure 2, also induced inflammation at its injection site (plantar injection) but the reaction was more transient and on the basis of the

**Table 3** Comparison of specific anti-inflammatory activity with content of bacterial endotoxin of four preparations of fraction B.

| | *Oedema test* | | Limulus *test* | | | | |
|---|---|---|---|---|---|---|---|
| | | | *Incubation time (min)* | | | | |
| *Sample* | *Activity/mg* | *mg/kg* | *5* | *10* | *15* | *20* | *Amount tested per 0.1 ml (μg)* |
| Non sterile | 0.59* | 9 | +++ | | | | 3.00 |
| Non sterile | 0.40* | 6.5 | +++ | | | | 6.70 |
| sterile | NS | 11 | — | + | ++ | +++ | 7.34 |
| sterile | NS | 15 | — | — | + | +++ | 8.15 |

The fractions B from four columns, run under non-sterile or sterile conditions were tested (s.c.) for anti-inflammatory activity by the rat paw kaolin oedema test and for endotoxin content by the *Limulus* amoebocyte lysate gelation test. The specific anti-inflammatory activity is expressed as in Table 1. The time and degree of gelation of the *Limulus* amoebocyte extract is dependent on the amount of endotoxin and this was estimated visually at the times indicated. +++=solid gel; ++=weak gel; +=increase in viscosity or opacity. Flocculation was taken as a negative result.

**Table 4** The growth of bacteria in column fractions

| | | Limulus *test* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Incubation time (min)* | | | | | |
| *Pool No.* | *No. viable bacterial cells/ml* | *5* | *6* | *7* | *8* | *9* | *10* |
| 1 | $10^3$ | — | — | — | + | ++ | ++ |
| 2 | $10^5$ | — | — | — | — | + | + |
| 3 | $10^8$ | — | + | ++ | +++ | +++ | +++ |
| 4 | $10^5$ | — | — | — | — | — | + |

Pools 1 to 4 were prepared as in Figure 2 under sterile conditions. Samples (75 ml) were innoculated with $10^5$ viable washed *E. coli* (1 ml) and incubated at 20°C for 3 days. Samples were taken for bacterial cell counting and for endotoxin testing by the *Limulus* test. Endotoxin determination results are shown as in Table 3 for a 1:100 dilution of the sample. By this dilution approximately 3 μg of fraction B (pool 3) was tested.

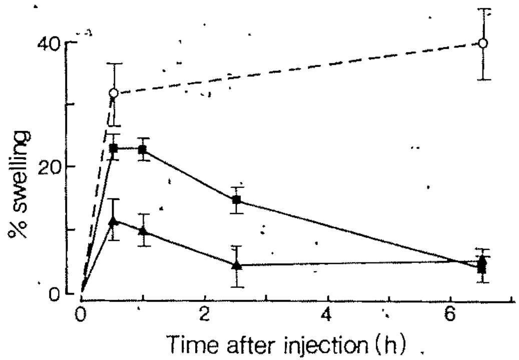

**Figure 3** The inflammatory effect of fractions A and B. Fraction A (0.1 ml, 200 mg/ml) in NaCl (○) or fraction B (0.1 ml, 10 mg/ml) (■ and ▲) were injected into the left foot pads of groups of 6 rats and the foot volumes measured at the times indicated. These are expressed as a percentage of the original volume. Vertical lines show s.e. Percentages are corrected for the volume due to 0.1 ml saline injection. One group (▲) had been pretreated with compound 48/80.

dose employed (1 mg), it had a more pronounced anti-inflammatory effect. In addition, doses which had a small but significant effect on the kaolin oedema caused no local inflammation of the injection site.

The pharmacological properties of fraction B were investigated in more detail. The local inflammation caused by fraction B was partially inhibited by pretreatment of the animals with compound 48/80 (Figure 3) and also by treatment with mepyramine maleate and methysergide bimaleate (2 mg/kg i.p.) together. Neither of these treatments antagonized the anti-inflammatory action of this fraction.

The effect of subcutaneous injection of fraction B on various pharmacological mediators was also measured. It caused a rapid increase in serum 11-hydroxycorticosteroid concentration from the untreated level of about 200 ng/ml, to 350 ng/ml which persisted for the duration of the experiment (6 hours).

Subcutaneous injection of fraction B caused no significant change in the serum haemolytic complement level of rats after 4 h, no change in the total number of circulating leucocytes and completely

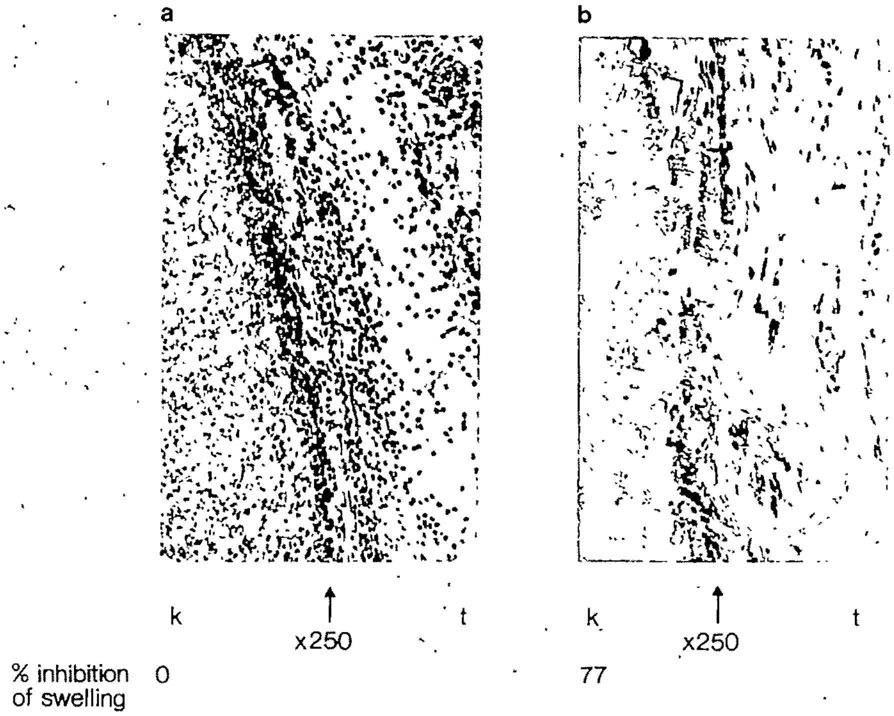

**Figure 4** Photomicrographs of sections of rat paws bearing kaolin oedemas: (a) shows part of the kaolin deposit and surrounding tissue of the paw of a control rat (treated with saline); (b) shows a similar area of a rat treated with 40 mg/kg fraction B subcutaneously. Arrow in each photograph shows the boundary between the kaolin deposit (k) with surrounding tissue (t).

abolished leucocyte infiltration into the kaolin injection site (Figure 4).

## Discussion

Our results confirm that freeze dried unfractionated rabbit anti-lymphocytic serum has anti-inflammatory activity. When the serum was fractionated by gel filtration under conditions where bacterial growth was minimized only one active fraction was obtained. It caused inflammation itself and may act via a counter-irritant mechanism (Laden, *et al.*, 1958; Atkinson & Hicks, 1971) and thus may not be a true anti-inflammatory factor.

When anti-lymphocytic serum was fractionated under conditions where bacterial growth occurred in spite of standard precautions, two active fractions were found. One fraction had similar properties to fraction A. The other had an apparently low molecular weight (less than 5000). This fraction (fraction B) was not dialysable and when rechromatographed its elution properties were consistent with a high molecular weight. Fraction B was found to contain Gram negative bacteria and when these bacteria were not present on the column, the anti-inflammatory activity did not appear. Thus the activity was generated by the action of bacteria.

Each species of bacteria found was therefore tested for anti-inflammatory activity in the kaolin oedema test and it was found that *E. coli* and *Ent. agglomerans* possessed activity. The lipopolysaccharide endotoxin of the former was extremely active and it is likely that this was one of the active principles.

The bacteria grow in and contaminate mainly the low molecular weight fraction. This is not surprising since it contains amino acids, small peptides, small carbohydrates and all the serum salts. It is, therefore, a more easily assimilated medium than mixtures of large proteins.

Our results show that, in spite of the recommended laboratory precautions (Pharmacia, 1970) for the prevention of microbial growth on columns, namely by constantly keeping the buffer flowing and occasionally washing with 0.02% sodium azide, it is still possible for column fractions to become contaminated. Some of the contaminating organisms were found to grow on agar in the presence of 1% sodium azide. Even after autoclaving the Sephadex, buffer and glassware and soaking the plastic parts of the column in disinfectant, it was still possible for some contamination to occur, but from Gram positive organisms, which were probably present in the laboratory atmosphere. These organisms were, however, found to be inactive in the kaolin oedema test.

The *Limulus* amoebocyte lysate gelation test is an extremely sensitive method for detecting the presence of bacterial endotoxin and has proved useful in our work. Concentrations of as little as 5 ng/ml can readily be detected. Unfortunately, it is too sensitive for the purposes of detecting whether a quantity of endotoxin will influence kaolin oedema formation and this necessitates large dilutions in endotoxin-free water. The commercial preparation of endotoxin which we have used has an $ED_{50}$ of approximately 25 µg/kg rat in the kaolin oedema test, which is nearly four orders of magnitude greater than the detection level of the *Limulus* test. The *Limulus* test is not strictly quantitative owing to the differing solubilities, the method of preparation of the endotoxin and the dependency on the species of bacteria. The rate of gelation and texture of the gel are however related to the concentration of a given endotoxin.

The shock reaction caused by endotoxin from Gram negative bacteria causes an increase in serum 11-hydroxycorticosteroid concentration (Munoz, 1961), activation of the complement system (Bladen, Gewurz & Mergenhagen, 1967) and an inhibition of leucocyte infiltration into areas of inflammation (Conti, Cluff & Scheder, 1961). Our fraction B caused both an increase in serum corticosteroid concentration and an inhibition of leucocyte infiltration. However, we found no evidence of complement depletion 4 h after injection of fraction B.

Fraction B also caused inflammation at its injection site. The rapid rate of formation and disappearance of this oedema suggested that 5-hydroxytryptamine and/or histamine were involved in this process. This hypothesis is supported by the finding that when amine release was prevented by previous administration of 48/80 or when methysergide and mepyramine were administered, the oedema was at least partially prevented. The relative contributions of histamine and 5-hydroxytryptamine were not investigated. Reduction of such oedema did not interfere with the ability of fraction B to inhibit oedema induced in the contralateral paw by injection of kaolin. Further, low doses of fraction B which did not cause measurable inflammation were still active against kaolin oedema. Therefore, the mode of action of fraction B is not through a counter-irritant mechanism.

Fraction B eluted from the Sephadex G-150 column at approximately 0.9 column volumes and has the same elution volume as the fraction C referred to by Billingham *et al.* (1970) in their fractionation of anti-lymphocytic serum. However, these workers, appear to have recycled some of the serum proteins over this peak, thus obscuring the presence of much low molecular weight material. It is, therefore, possible that the activity which they found in their fraction C is the same as that described by us as fraction B. Also, fraction B of Billingham *et al.* (1970) is probably the same as our fraction A, since these have the same elution volumes (approximately 0.4 column volumes).

Billingham *et al.* (1970) did not regard this material as being a true anti-inflammatory substance in view of its immunosuppressive and lymphocytotoxic properties.

The active material isolated from normal human serum (Ford-Hutchinson, Insley, Elliott, Sturgess & Smith, 1973) was isolated by gel filtration as two low molecular weight fractions from the edges of an optical density peak, the central portion of which could not be tested because it proved toxic to rats. An injection of bacterial endotoxin would have the same result if sufficient were injected. The human fraction appears to act by preventing leucocyte migration but not by depleting complement (Walker, Smith, Ford-Hutchinson & Billimoria, 1975).

Léme & Schapoval (1975), in their work with inflamed paw perfusates have been able to show oedema inhibiting activity when the perfusate was injected into the rats. Concomitant with this they observed an increase in serum corticosterone concentration.

None of these authors has reported taking any precautions to exclude contamination by bacteria nor have they attempted to measure any endotoxin which may have been present. The pharmacological properties of these anti-inflammatory fractions are similar to those of our endotoxin containing fraction B.

It is clear from our work that where experiments have been carried out under conditions which exclude significant bacterial contamination, no characterizable low molecular weight anti-inflammatory substances could be found in anti-lymphocytic serum.

We thank Mr A. Turnbull for doing the rat paw oedema experiments, Mr M. Heavens for the bacteriological work and helpful suggestions and discussions, Mr P. Boon for providing facilities for the bacteriology, Mr R. Christian for the microscopy and Mrs P. Rham, Superintendant Radiographer at Horsham Hospital for providing X-ray facilities.

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*(Received August 23, 1976.*
*Revised September 26, 1976.)*

*Br. J. Pharmac.* (1977), 59, 269–273

# VASOCONSTRICTOR RESPONSE TO ARACHIDONIC ACID IN THE ISOLATED HIND LIMB OF THE DOG

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1 Arachidonic acid (AA) (25–200 μg/kg) produced a dose-related increase in perfusion pressure in dog isolated hind limbs perfused with either blood or a platelet-free perfusate.

2 Prostaglandin $E_2$ produced vasodilatation while prostaglandin $F_{2\alpha}$ produced no vascular change at these administered doses.

3 Phentolamine did not alter the arachidonic acid response, eliminating possible $\alpha$-adrenoceptor mediation.

4 Aspirin and indomethacin blocked the vasoconstrictor response to AA.

5 This study indicates that a vasoactive intermediate in prostaglandin synthesis can be elaborated in the absence of platelets.

## Introduction

Arachidonic acid (AA), the precursor of the bisenoic prostaglandins $E_2$ and $F_{2\alpha}$, consistently produces a systemic hypotensive response when administered intravenously to cats (Jaques, 1959), rats (Cohen, Sztokalo & Hinsch, 1973), rabbits (Ichikawa & Yamada, 1962) and dogs (Rose, Johnson, Ramwell & Kot, 1974). Evidence has been presented that suggests the hypotensive response is produced by one or more potent vasoactive intermediates in the pathway leading to prostaglandin synthesis (Rose *et al.*, 1974).

Hamberg, Hedqvist, Strandberg, Svensson & Samuelsson (1975) have shown that intermediates in prostaglandin synthesis constrict the pulmonary arteries in cats. This observation implies that the initial hypotensive response is due to decreased return to the left ventricle and decreased cardiac output. These authors have suggested that conversion of a vasoconstrictor intermediate to its degradation products, particularly prostaglandin $E_2$, produces a longer lasting reduction in blood pressure due to peripheral vasodilatation. The well-known enhancement of platelet aggregation by AA (Silver, Hoch, Kocsis, Ingerman & Smith, 1974) suggests that another possible mechanism for acute systemic hypotension could be momentary obstruction of the pulmonary vasculature.

The responses of systemic blood vessels to AA are thus not demonstrable in whole animal preparations which reveal only the result of a complex interplay of central and peripheral effects. The purpose of the present study was to isolate a peripheral regional component of the hypotensive response to AA. The dog hind limb was used to investigate this potentially important phenomenon.

## Methods

Mongrel dogs of either sex (13 to 26 kg in body weight) were anaesthetized with sodium pentobarbitone (30 mg/kg) and intubated with a cuffed endotracheal tube. The right femoral artery was catheterized for systemic arterial pressure recording. The right femoral vein was catheterized to withdraw blood for the extracorporeal circuit. The left hind limb circulation was isolated from the remainder of the systemic circulation. The sciatic nerve was left intact. The superficial limb musculature was severed and an adjustable metal band was tightened around the femur to prevent any cross-circulation via the deep musculature.

Autologous blood anticoagulated with heparin (8 mg/kg) was collected in a reservoir and maintained at 37°C. With a peristaltic pump (Sigma), blood from the reservoir was pumped at a constant flow rate (3 ml $kg^{-1}$ $min^{-1}$) through the femoral artery catheter, circulated through the limb, and via the venous catheter to a small oxygenator (Temptrol). A mixture of 98% air and 2% $CO_2$ was used to aerate the blood and maintain arterial blood gases and pH in the physiological range. The blood was returned to the reservoir to be reperfused. Arterial and venous pressures in the isolated limb were monitored at their respective catheters. All test substances were administered in random sequence. Each agent was administered by bolus injection into the tubing between the pump and arterial supply to the limb. Control injections of vehicle solution produced no injection artifact. Tracer studies using radioiodinated serum albumin demonstrated no cross-circulation between the closed limb circuit and the systemic circulation.

The sodium salts of arachidonic acid (5,8,11,14-eicosatetraenoic acid), >99% pure from porcine liver (Sigma & Nu-Chek), and linoleic acid (99% Sigma) were prepared by dissolving them with sodium carbonate (100 mM) during constant stirring under $N_2$, in the absence of light. The resulting solutions were water clear and used only on the day of preparation. Prostaglandins were generously provided by The Upjohn Co. Solutions of prostaglandin (1 mg/ml) in ethanol were evaporated to dryness under $N_2$. The residue was dissolved in 0.9% w/v NaCl solution (saline).

In two animals, phentolamine (250 μg/kg body weight) was introduced into the isolated hind limb to block α-adrenoceptors. Both aspirin (25 mg/kg) and indomethacin (2.5 mg/kg) were used to block prostaglandin synthetase enzymes. Acetylsalicylic acid powder was dissolved in modified Tyrode solution and pH was adjusted to 7.35 with sodium hydroxide. Indomethacin (2 mg/ml) was made by combining a solution of 50 mg indomethacin in 5 ml distilled water with 15.6 mg $Na_2CO_3$ in 5 ml distilled water. This solution was mixed for 10 min, diluted to a final volume of 25 ml, and filtered (pH 7.74).

In platelet-free studies, the isolated limb was perfused with Perfudex (Pharmacia), a 5% dextran electrolyte solution with $HCO_3^-$ (25 mM) and $CaCl_2$ (25 mM) added. Platelet counts of the effluent indicate that 85% of the original platelets ($2.3 \times 10^5/mm^3$) were eliminated by this procedure. The effluent was not recirculated but drained off in order to eliminate any trapped platelets released from the vascular bed.

## Results

Increasingly larger doses of AA were administered to seven hind limb preparations to establish a dose-response curve (Figure 1). The percentage increase in mean limb arterial perfusion pressure with doses of 25, 50, 100, 150 and 200 μg/kg were 3.1 ± 1.6 (s.e.), 11.5 ± 3.9, 17.9 ± 2.9, 20.9 ± 3.8 and 19.9 ± 6.6%, respectively. Two trials at 400 μg/kg resulted in a mean increase of 18.0%.

An example of the vasoconstrictor effect of AA (150 μg/kg) in the isolated limb, is shown in Figure 2. The isolated limb arterial pressure rose substantially above the control values in an average time-to-onset of seven seconds. At the same time, the systemic arterial pressure and limb venous pressure remained the same. A similar response was produced by noradrenaline (1 μg/kg). Phentolamine completely blocked the pressor response to noradrenaline, but did not alter the vasoconstrictor response to AA.

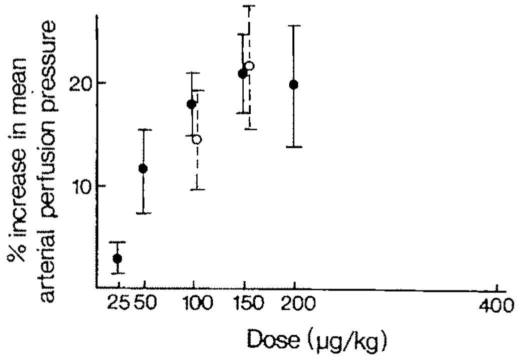

**Figure 1** Percentage increase in mean arterial perfusion pressure as a function of the dose of arachidonic acid administered to the isolated hind limb preparation of the dog: (●) represent values obtained with autologous blood as perfusate ($n=7$); (○) are values with a platelet-free perfusate ($n=3$). Vertical lines show s.e. means.

In three platelet-free preparations doses of 100 and 150 μg/kg of AA were administered in the same manner. Rapid increases in perfusion pressure were again observed. The percentage changes in mean femoral arterial pressure were 14.6 ± 4.7 and 21.6 ± 7.1%. Blood perfusion experiments in the same animals showed increases of 17.9 ± 2.9 and 20.9 ± 3.8% respectively for the same doses (Figure 1).

Prostaglandin $E_2$ and $F_{2\alpha}$ were administered in a dose range that consistently elicited a systemic response in the intact dog (1–6 μg/kg). Initial administrations of prostaglandin $E_2$ produced marked vasodilatation. However, subsequent doses in the same limb produced no response. Prostaglandin $F_{2\alpha}$ had no effect on limb perfusion pressure. Periodic monitoring of arterial blood gases indicated values within the physiological range ($PaO_2$ 138.1 ± 6.7, $PaCO_2$ 26.4 ± 0.9, pH 7.35 ± 0.02). Linoleic acid, used as a control fatty acid, was administered in a dose of 150 μg/kg. No vascular response was produced. Both aspirin ($n=3$) and indomethacin ($n=5$) induced a transient vasodilatation in which the perfusion pressures returned to control values within 1 minute. These synthetase inhibitors completely blocked the vascular response to AA in the isolated limb.

## Discussion

Earlier studies have shown that both AA and prostaglandin $E_2$ produce a systemic hypotensive

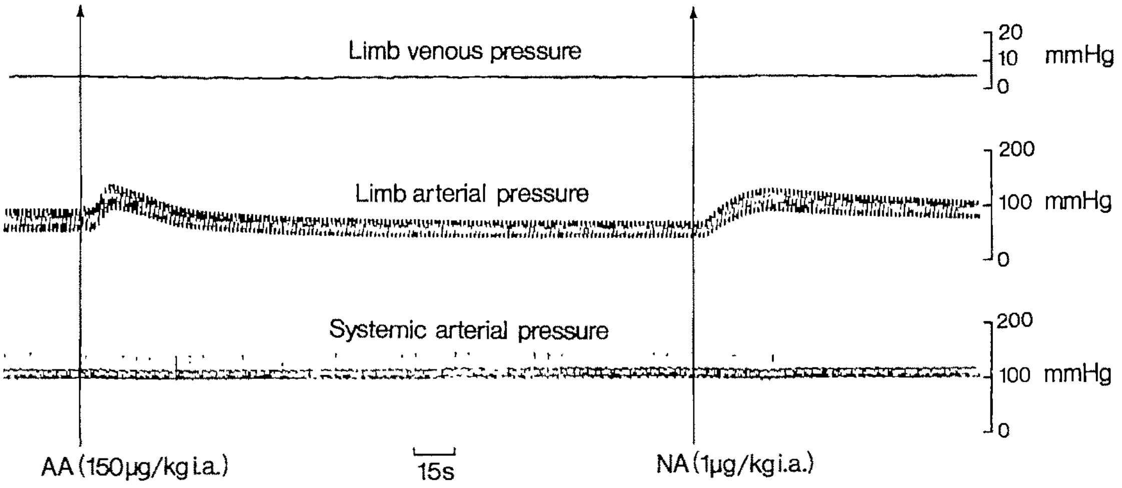

**Figure 2** Effects of arachidonic acid (AA, 150 µg/kg) and noradrenaline (NA, 1 µg/kg) on femoral venous pressure, femoral arterial pressure and systemic arterial pressure in the isolated hind limb preparation of the dog. Arrows indicate points of intra-arterial injection.

response in dogs. However, they differ in cardiac and pulmonary vascular effects (Rose *et al.*, 1974; Wicks, Rose, Johnson, Ramwell & Kot, 1976).

In order to investigate the more direct effects of AA on a representative systemic vascular bed, the isolated hind limb preparation of the dog was selected for study. The systemic hypotensive response observed in intact animals and the results of AA infusion studies by Ryan & Zimmerman (1974) suggested that there would be a marked decrease in perfusion pressure in the limb due to a decrease in arterial resistance. The opposite was the case. AA caused the isolated limb perfusion pressure to rise substantially above the control values, indicating a transient increase in limb vascular resistance.

The possibility that the vascular response to AA is mediated by the release of endogenous noradrenaline or direct stimulation of $\alpha$-adrenoceptors is unlikely since phentolamine administration did not modify this response. Furthermore, pulmonary vasoconstriction produced by AA, similar to the response in the isolated hind limb, was not affected by $\beta$-adrenoceptor, cholinoceptor or 5-hydroxytryptamine antagonists (Wicks *et al.*, 1976). These data suggest that other receptors must be present that mediate the vasoconstriction induced by AA.

The increase in perfusion pressure could be explained by platelet aggregation at arteriolar and capillary sites. AA has been conclusively shown to induce platelet aggregation both *in vitro* (Silver, Smith, Ingerman & Kocsis, 1973; Vargaftig & Zirins, 1973; Rose *et al.* 1974) and *in vivo* preparations (Silver *et al.*, 1974; Furlow & Bass, 1975) in several different species. The results of experiments using the platelet-free perfusate strongly suggest that the increased perfusion pressure is independent of the release of vasoactive platelet products or mechanical obstruction of small vessels by platelet aggregates.

Clearly, some product in the biosynthetic pathway from AA to the bisenoic prostaglandins is vasoconstrictor in the canine hind limb. Both prostaglandin $E_2$ and $F_{2\alpha}$ were administered in a dose range that consistently elicits a systemic response in the intact dog. The initial injections of prostaglandin $E_2$ produced vasodilatation of the hind limb which is in agreement with Kadowitz (1972) and others. The failure to elicit a response upon subsequent injections of prostaglandin $E_2$ may be due to the limb vasculature being maximally dilated as suggested by Daugherty (1971). In contrast to the studies of Ducharme, Weeks & Montgomery (1968) and Greenberg & Sparks (1969), prostaglandin $F_{2\alpha}$ did not induce vasoconstriction in the isolated hind limb. However, the techniques employed by these investigators were substantially different. Brody & Kadowitz (1974) suggest that low concentrations of prostaglandin $F_{2\alpha}$ may not exert an effect on the vascular resistance of the limb.

The vascular responses to AA in the isolated hind limb were blocked by aspirin and indomethacin, synthetase inhibitors of the endoperoxide intermediates (Samuelsson & Hamberg, 1974; Malmsten, Hamberg, Svensson & Samuelsson, 1975). Therefore, vasoconstriction is not a direct effect of AA. Linoleic acid produced no vascular response indicating that the increased perfusion pressure is not due to a non-specific fatty acid effect.

These findings suggest that the substance producing the increase in vascular resistance is not AA, or prostaglandin $E_2$ or $F_{2\alpha}$, but a vasoactive intermediate. Endoperoxide intermediate compounds have been isolated by Nugteren & Hazelhof (1973) and Hamberg, Svensson, Wakabayashi & Samuelsson (1974). Although these substances are unstable, they are more potent vasoconstrictors than their respective prostaglandins (Hamberg *et al.*, 1975). Thromboxane $A_2$, a further metabolite of AA, also has vasoactive properties (Kolata, 1975; Tuvemo, Strandberg, Hamberg & Samuelsson, 1976). These compounds are possible alternatives to AA and prostaglandins $E_2$ and $F_{2\alpha}$ as vasoactive substances responsible for the observed increases in hind limb vasoconstriction.

A discrepancy exists between the systemic hypotensive response to AA in the intact dog and the vasoconstrictor response to AA in the isolated limb. Although a reduction in cardiac output was suggested by Hamberg *et al.* (1975) as a possible explanation for the systemic response to AA, the hypothesis does not appear likely. Ascending aortic blood flow measured during systemic administration of AA showed little change or an increase in cardiac output. Vasodilatation in the splanchnic circulation (Anggard & Larsson, 1974) may in a large part account for the systemic hypotensive response.

The authors gratefully acknowledge the technical assistance of Ms Teresita Maish and Mr Israel Alter in this investigation. This work was supported by U.S.P.H.S. Training Grant HL-05527 and a grant from the Educational Foundation of America.

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*(Received June 8, 1976.*
*Revised September 28, 1976.*

*Br. J. Pharmac.* (1977), 59, 275–281

# EFFECTS OF THYROIDECTOMY AND L-THYROXINE ON ADRENALINE AND NORADRENALINE CONCENTRATIONS IN THE ADRENAL GLANDS AND PLASMA OF RATS DURING THE PRO-OESTROUS PHASE OF THE OESTROUS CYCLE AND PREGNANCY

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1 The influence of thyroidectomy upon the adrenaline and noradrenaline content of adrenal glands and plasma in mature female rats in pro-oestrus and in pregnant rats was studied.

2 Adrenal adrenaline and noradrenaline declined significantly after thyroidectomy in pro-oestrous and pregnant females but the effects were more marked in pregnant females.

3 Plasma adrenaline increased by 160% after thyroidectomy in pro-oestrous females but similar treatment resulted in 85% decrease in plasma adrenaline of pregnant rats. The loss of thyroid increased plasma noradrenaline significantly in both groups of females.

4 The administration of L-thyroxine to thyroidectomized females increased adrenal noradrenaline stores of both the groups. The effects of L-thyroxine in pro-oestrous females resulted in decreased adrenaline stores of the adrenals but the pregnant group showed an increase. Plasma noradrenaline increased after treatment of pro-oestrous and pregnant-thyroidectomized females with L-thyroxine.

5 The thyroidectomized females in pro-oestrous phase receiving L-thyroxine showed a return to the control values for plasma adrenaline but in pregnant females whose plasma adrenaline had declined after thyroidectomy no such change occurred.

6 Considering the variations in total catecholamines in plasma and adrenals, it was observed that the loss of thyroid hormones results in an increase in total catecholamine storage and output in the blood. The results provide evidence that the thyroid-catecholamine interrelation is significantly affected by pregnancy.

## Introduction

The influence of endocrine gland secretions upon the processes of monoamine synthesis, release and metabolism has been a subject of interest during the past decade (Axelrod, Mueller & Thoenen, 1970; von Euler, 1972). More recently the biochemical mechanisms that may stimulate or inhibit the synthesis or breakdown of the monoamines under the influence of different hormones have been examined at the molecular level (Wurtman, Pohorecky & Baliga, 1972). The fundamental work on the effects of thyroid hormones on regulation of monoamines was provided by Wurtman, Kopin & Axelrod (1963) and Landsberg & Axelrod (1968), who analysed the relationship between thyroid function and the cardiac disposition of catecholamines. The latter authors found that thyroid hormones can influence both monoamine oxidase and catechol-*O*-methyltransferase since a few minutes after a dose of adrenaline, the amount found in the hyperthyroid rat heart was considerably smaller than in the normal heart. A study of the tritiated metabolites formed from tritiated adrenaline *in vivo* on the hyperthyroid rat heart showed a decrease in the cardiac content of all the main metabolites, while they were increased in hypothyroidism (Landsberg & Axelrod, 1968). Most of these studies have been confined to normal animals and little attention has been given to the effect of hormones on monoamine regulation during hormonal deficiencies. Recently, evidence has been provided that the effects of drugs and hormones on monoamine metabolism are greatly modified during various physiological states such as pregnancy and the oestrous cycle (Parvez, Parvez & Youdim, 1975; Parvez & Parvez, 1976; Parvez, Parvez, Raza-Bukhari & Youdim, 1976). The present experiments were performed to study the influence of thyroidectomy during pregnancy and in pro-oestrus upon the storage and release of adrenaline and noradrenaline from adrenal gland into plasma. Thyroidectomized animals were also given L-thyroxine to examine the effects of this hormone on the two parameters studied.

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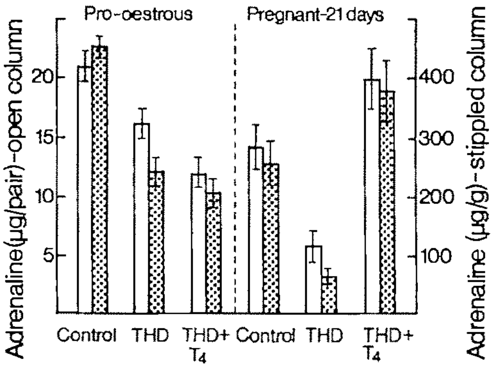

**Figure 1** The influence of thyroidectomy and L-thyroxine administration (s.c.) upon adrenaline storage in adrenal gland of pregnant rats and rats during pro-oestrous phase of the oestrous cycle. The results are expressed in µg/pair (open columns) as well as µg/g (stippled columns) of adrenals. Control (non-operated rats on 15th day of pregnancy or during pro-oestrus), THD (thyroidectomized) and THD + $T_4$ (thyroidectomized rats receiving L-thyroxine s.c.). Each group contained 8 to 16 rats and each column represents mean result, vertical lines show s.e. mean.

Significance of differences between groups:

| | *Pro-oestrus* | *Pregnant* |
|---|---|---|
| Control *vs* THD | $P < 0.01$ (µg/pair) | $P < 0.001$ (µg/pair) |
| | $P < 0.001$ (µg/g) | $P < 0.001$ (µg/g) |
| THD *vs* THD+$T_4$ | $P < 0.02$ (µg/pair) | $P < 0.001$ (µg/pair) |
| | | $P < 0.001$ (µg/g) |

## Methods

Albino female rats of Sherman strain obtained from Janvier, Paris were used in all experiments. The females weighed 250 ± 50 g (range) and were housed at a constant temperature of 21 °C with natural day and night cycles. They were fed with commercial laboratory food *ad libitum*. The females were impregnated by keeping one male in the cage of seven females from 18 h 00 min to 09 h 00 min only once. The fertilization was assumed to occur at 02 h 00 min. Thus, it was possible to make an estimation for an approximation of pregnancy ± 7 hours. The females were palpated abdominally 14 days later for the verification of pregnancy. Under normal conditions parturition took place during Days 21.5 and 22 of gestation (Parvez, Gripois & Parvez, 1974).

### *Thyroidectomy and L-thyroxine administration*

The animals were thyroidectomized on Day 15 of pregnancy or during the well defined pro-oestrous phase of the oestrous cycle. The operation was performed under ether anaesthesia according to the technique of Zarrow, Yochim, McCarthy & Sanborn (1964). All the operated rats were examined for the effectiveness of thyroidectomy by measuring their weights 3 days after the operation. The administration of L-thyroxine (Nutritional Biochemicals) started on the day after operation and lasted for five days. All the thyroidectomized rats received 3 µg/100 g body weight of L-thyroxine daily at 10 h 00 min. On the sixth day, the animals were killed by neck fracture and the blood taken and adrenals excised in less than 1 minute. A maximum of 3 ml of blood was taken from the abdominal aorta of each rat.

### *Assay of adrenaline and noradrenaline in plasma*

Heparinized blood from 5 rats was pooled in a glass centrifuge tube containing 2 ml of potassium fluoride (2%) and 2 ml of potassium thiosulphate (3%). The blood was centrifuged at 1 °C for 10 min at 10,000 rev/minute. Adrenaline and noradrenaline in plasma were isolated by adsorption on acid activated aluminium oxide (von Euler & Lishajko, 1959). The batch process was preferred to columns since the recoveries were better (Anton & Sayre, 1962). One g of alumina was washed twice with quartz distilled water and added to the 2 ml of plasma in a centrifuge tube. The pH was adjusted to 8.4 and the adsorption allowed to continue with gentle agitation for 4 minutes. The tubes were then centrifuged at 3000 *g* for 1.5 min and the supernatant discarded. The alumina was washed with 20 ml of distilled water and the catecholamines eluted with 4 ml of 0.25 N acetic acid. The trihydroxindole procedure was employed for simultaneous estimation of both adrenaline and noradrenaline.

The mean values are given together with the standard errors of the means, and statistical comparison made using Student's *t* test.

## Results

### *Effect of thyroidectomy on adrenal gland adrenaline*

Figure 1 illustrates the influence of thyroidectomy and compensatory treatment with L-thyroxine upon

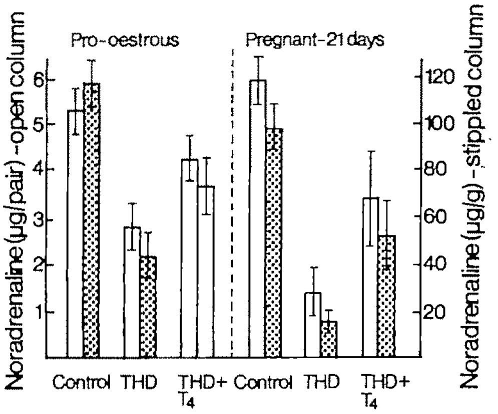

**Figure 2** Noradrenaline storage in adrenals of females thyroidectomized during pro-oestrous phase or on 15th day of pregnancy. THD = thyroidectomized rats, $T_4$ = -thyroxine given subcutaneously. Each group represents mean of 8 to 16 values; vertical lines show s.e. mean.
Significance of differences between groups:

| | *Pro-oestrus* | *Pregnant* |
|---|---|---|
| Control *vs* THD | $P<0.001$ (µg/pair)<br>$P<0.001$ (µg/g) | $P<0.001$ (µg/pair)<br>$P<0.001$ (µg/g) |
| THD *vs* THD + $T_4$ | $P<0.01$ (µg/pair)<br>$P<0.01$ (µg/g) | $P<0.02$ (µg/pair)<br>$P<0.02$ (µg/g) |

adrenaline storage in the adrenal gland of female rats during the pro-oestrous phase of the oestrous cycle and in late pregnancy. The results have been expressed in µg/pair of adrenal glands as well as in µg/g of adrenal weight. The adrenaline content of the adrenal gland in the pregnant rats was lower than in the controls ($P<0.01$), confirming earlier results (Parvez *et al.*, 1976) obtained with rabbits. The removal of the thyroid gland resulted in a significant decrease in the adrenaline content of the adrenal glands in both groups of females. The decrease was 32% (µg/pair) and 55% (µg/g) for adrenals of the rats in pro-oestrus, and in the pregnant group it was 63% (µg/pair) and 74% (µg/gram). Compensatory treatment of pro-oestrous females with L-thyroxine further reduced the adrenaline stores in the adrenal gland but the pregnant females responded differently to this treatment by showing an increase, 355% (µg/pair) and 567% (µg/gram). The mean values in thyroidectomized females receiving L-thyroxine during pregnancy were close to the control values.

### *Effect of thyroidectomy on adrenal gland noradrenaline*

The storage of noradrenaline in the adrenal gland of female rats thyroidectomized during the pro-oestrous phase of the oestrous cycle and on 15th day of pregnancy is shown in Figure 2. Thyroidectomy resulted in a decrease of 47% (µg/pair) and 63% (µg/g) when compared with values of control female rats in the pro-oestrous phase, and in a 77% and an 84% decrease (µg/pair and µg/g respectively) in the adrenal noradrenaline content of the pregnant group when compared with non-operated pregnant rats. The administration of L-thyroxine to thyroidectomized females in both groups of rats resulted in an increased storage of noradrenaline and the mean values showed a tendency towards the non-operated values. The extent of noradrenaline increase in pregnant females was greater than in pro-oestrous females.

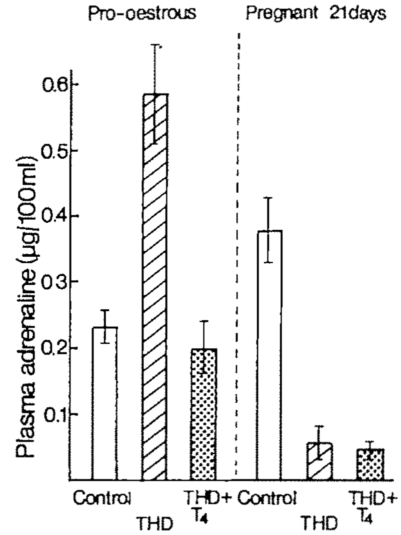

**Figure 3** Variations in plasma adrenaline after thyroidectomy and compensatory treatment of L-thyroxine to cyclic or pregnant rats. Each group represents mean of 16 to 18 females; vertical lines show s.e. mean. THD = thyroidectomized, and THD + $T_4$ = thyroidectomized rats receiving L-thyroxine ($T_4$).
Significance of difference between the groups:

| | *Pro-oestrus* | *Pregnant* |
|---|---|---|
| Control *vs* THD | $P<0.001$ | $P<0.001$ |
| THD *vs* THD + $T_4$ | $P<0.001$ | NS |
| Control *vs* THD + $T_4$ | NS | $P<0.001$ |

### *Effect of exogenous thyroxine on the plasma concentrations of adrenaline and noradrenaline*

The variations in plasma concentrations of adrenaline after removal of the thyroid gland in female rats during pro-oestrus and on Day 15 of pregnancy are shown in Figure 3. After thyroidectomy the adrenaline content of plasma in pro-oestrous females increased by 160%. The removal of the thyroid gland in the pregnant rats produced an opposite effect; the adrenaline content of plasma fell by 85%. The treatment of thyroidectomized pro-oestrous rats with

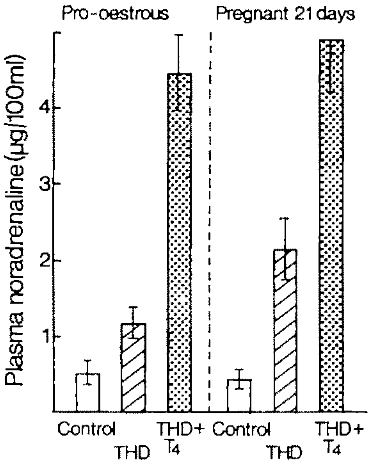

**Figure 4** Plasma noradrenaline content in thyroidectomized female rats operated during oestrous cycle or on day 15 of pregnancy. THD = thyroidectomized; $T_4$ = L-thyroxine treated. Each group is mean value from 18 rats; vertical lines show s.e. mean.

Significance of difference between the groups:

| | *Pro-oestrus* | *Pregnant* |
|---|---|---|
| Control *vs* THD | $P<0.01$ | $P<0.001$ |
| THD *vs* THD + $T_4$ | $P<0.001$ | $P<0.001$ |

L-thyroxine lowered the adrenaline content of plasma but the mean value was not significantly different from that of controls. L-Thyroxine administration to pregnant thyroidectomized rats did not produce any change in plasma adrenaline and the mean value remained significantly lower than in the non-operated pregnant rats.

Figure 4 provides a comparison of plasma noradrenaline in pro-oestrous and pregnant rats after thyroidectomy and compensatory treatment with L-thyroxine. The removal of the thyroid gland resulted in 125% increase in plasma noradrenaline of females during the pro-oestrous phase of the oestrous cycle. The effect of thyroidectomy in pregnant females was similar to that in pro-oestrous females but the increase in noradrenaline content of plasma was much higher (393% higher than control value). The administration of L-thyroxine to rats in pro-oestrous phase resulted in a marked increase in plasma noradrenaline content. Similar treatment of pregnant thyroidectomized females with L-thyroxine also produced a significant increase in the concentration of the transmitter in the plasma.

The variations in total catecholamine (adrenaline + noradrenaline) content of plasma and adrenal gland after thyroidectomy as well as after compensatory treatment with L-thyroxine in pregnant and pro-oestrous animals are shown in Table 1. The per cent changes from non-operated controls in both the groups are also indicated. These parameters have been calculated to show that the effects of thyroid deficiency during normal life and pregnancy differ to a great extent for the storage of catecholamines in the adrenal medulla. The changes in total plasma amine levels which have been observed suggest that each catecholamine changes greatly without modifying the total catecholamine concentration. The response to L-thyroxine administration in thyroidectomized females during pregnancy was always greater than that in rats in the pro-oestrous phase of the oestrous cycle. These results show that hormonal sensitivity changes considerably during pregnancy for the processes of monoamine storage and release.

## Discussion

In addition to many other well known effects upon endocrine metabolism, removal of the thyroid gland influences the storage and release of adrenaline and noradrenaline from the adrenal gland during the pro-oestrous phase of the oestrous cycle as well as during pregnancy. The present results show that the noradrenaline content of the adrenal gland declines after thyroidectomy in both pro-oestrous and pregnant animals by 47% and 84% respectively, whereas at the same time a significant increase in plasma noradrenaline concentration in both the groups of animals occurs which would suggest that there is an accelerated release of noradrenaline from the adrenal gland into the circulation. Previous study of the role of thyroid hormones on noradrenaline turnover and metabolism in the rat heart suggests that thyroid deficiency is associated with a significant decrease in endogenous noradrenaline content of the heart, possibly mediated by modifications in sympathetic nervous activity (Landsberg & Axelrod, 1968). During hypothyroidism in the rat the activity of adrenal gland monoamine oxidase is only 55% of the normal value in the male rat but in the female there is no change (Wurtman *et al.*, 1963). This observation leads to the suggestion that intracellular degradation of monoamines affected by monoamine oxidase remains unchanged in the hypothyroid female rat and the marked increase in plasma noradrenaline is mainly caused by accelerated release from the adrenal gland. Moreover it was also found that the activity of enzyme catechol-*O*-methyltransferase was unaffected by removal of the thyroid gland and these effects were similar in male and female rats (Wurtman *et al.*, 1963). This evidence is in agreement with the present results since the rate of degradation of plasma noradrenaline remained at a steady state after thyroidectomy and the increased release from the adrenal gland can be detected in the blood.

Adrenaline in the adrenal glands of pro-oestrous and pregnant females reacted in a similar manner to

**Table 1** Variations in total catecholamines (adrenaline (Ad) + noradrenaline (NA)) in adrenal gland and plasma of female rats thyroidectomized during pro-oestrous phase of the oestrus cycle or on the day 15 of pregnancy. The effects of L-thyroxine ($T_4$) administered for 5 days (3 µg/100 g body weight daily) are also shown.

| *Treatment* | *Adrenal Ad + NA (µg/pair)* | *% of non-operated controls* | *Adrenal Ad + NA (µg/g)* | *% of non-operated controls* | *Number of cases* | *Plasma Ad + NA (µg/100 ml)* | *% of non-operated controls* | *Number of cases* |
|---|---|---|---|---|---|---|---|---|
| Thyroidectomized in pro-oestrus | 19 ± 1.7 | 73 | 298 ± 33.5 | 52 | 10 | 1.75 ± 0.28 | 233 | 16 |
| Thyroidectomized in pregnancy | 7.1 ± 1.84 | 34 | 83 ± 20.7 | 23 | 13 | 2.2 ± 0.41 | 275 | 18 |
| Thyroidectomized in pro-oestrus + $T_4$ | 16.2 ± 1.7 | 62 | 280 ± 35.4 | 48 | 8 | 4.6 ± 0.5 | 613 | 16 |
| Thyroidectomized in pregnancy + $T_4$ | 23.8 ± 3.6 | 112 | 431 ± 64 | 120 | 8 | 4.93 ± 0.69 | 616 | 16 |

The % values have been calculated from non-operated mean value of each respective group.

Thyroidectomized *vs* Thyroidectomized + $T_4$

Significance of differences between the groups:

*Pregnant*
Adrenals µg/g and µg/pair ($P < 0.001$)
Plasma ($P < 0.002$)

*Pro-oestrus*
Adrenals (NS)
Plasma ($P < 0.001$)

its precursor noradrenaline since both groups showed a significant decline from normal values after thyroidectomy. The pro-oestrous females showed a significant increase in plasma adrenaline after thyroidectomy but the pregnant females had a lowered plasma adrenaline concentration after removal of the thyroid gland. This difference can be explained on the basis of modifications in endocrine status of pregnant females which is affected by the state of gestation. It is well established that metabolic effects mediated by the thyroid gland are greatly influenced during pregnancy (Furth & Pagliara, 1971). The thyroxine binding globulin capacity increases in pregnancy 2.5-fold (Robbins & Rall, 1967). Serum from pregnant animals also has a subnormal thyroxine binding pre-albumin concentration (Woeber & Ingbar, 1968). Suppression or augmentation of hormonal activity by thyroxine binding pre-albumin or thyroxine binding globulin is altered during pregnancy mainly as a result of reduction in chemical activity of steroid hormones (Westphall, 1970). The rise in thyroxine binding serum proteins during pregnancy has been recently suggested to be a consequence of oestrogen stimulation and this effect can also be seen in non-pregnant rats receiving oestrogen treatment (Furth & Pagliara, 1971). This rise in the binding capacity of protein results in an increased concentration of plasma thyroxine (Nikitovitch & Knobil, 1955). Because the binding capacity of serum protein is increased in pregnancy, the renin uptake is decreased (Sterling & Brenner, 1966). Considering evolutionary changes in the ratio of free and bound corticosteroids, oestrogens and progesterone, one can easily suggest that thyroid function which is more or less dependent on the normal function of the above hormones is certainly affected by the fluctuations which take place in these hormones during pregnancy (Wiest, Kidwell & Balough, 1968; Lau, Liao & Petropoulos, 1971; Shaikh, 1971). All these hormones have been shown to have a direct effect on the enzymes of monoamine metabolism (Phorecky & Wurtman, 1971; Parvez & Parvez, 1973; Holzbauer & Youdim, 1973; Parvez *et al.*, 1975; Parvez *et al.*, 1976). Therefore any consequent change in corticosteroids, oestrogens or progesterone possibly induced by thyroid deficiency should directly affect the plasma level of adrenaline in pregnant rats. Another piece of evidence which should also be considered is that the percentage augmentation of noradrenaline in the adrenal glands is much greater in pregnant rats. Therefore very little adrenaline appears to be formed from the noradrenaline still present in the adrenals, and which can eventually be released from the gland. These modifications appear to be related to the differences observed between pregnant and cyclic animals. In pregnant rabbits the adrenaline content of adrenal gland declines throughout the gestational period and appears to be related to the fall in the activity of the enzyme phenylethanolamine-*N*-methyltransferase (Parvez *et al.*, 1976).

The effect of thyroidectomy upon noradrenaline storage in the adrenals and its plasma concentration was similar in pro-oestrous and pregnant rats. The increased plasma noradrenaline after removal of thyroid and administration of L-thyroxine confirms the previous observations of von Euler (1954) that adrenal secretion has very little influence on blood noradrenaline. It appears that deprivation of thyroid hormones stimulates sympathetic nerve terminals which liberate or synthesize more noradrenaline. It has been shown that monoamine synthesis in brain and peripheral neurones increases in hypothyroidism and decreases in hyperthyroidism (Prange, Meek & Lipton, 1970). The high levels of plasma noradrenaline observed in thyroidectomized rats after thyroxine treatment suggests that the dose employed was not large enough to restore the transmitter concentration to its normal levels. It can be seen (Table 1) that the total catecholamines in the adrenals were only slightly modified after the injections of thyroxine but plasma concentrations increased. This observation suggests an activation of adrenergic nerve terminals which can eventually release excessive amounts of noradrenaline.

In conclusion our results provide evidence that thyroid monoamine interrelations are modified to a great extent during pregnancy. The treatment of thyroid deficiency with L-thyroxine has a qualitatively different effect in the normal and pregnant animals.

This study was supported by DGRST, France, under grant No. 650919. The generous help of Dr Raynaud of CEA, and Abid Raza of Paris University is gratefully acknowledged. We are grateful to Miss Danielle Telliez for providing technical assistance in some of the experiments.

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*(Received June 10, 1976.*
*Revised August 20, 1976)*

*Br. J. Pharmac.* (1977), 59, 283–292

# DOPAMINE-INDUCED NEUROGENIC VASODILATATION IN THE INTACT HINDLEG OF THE DOG

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**1** The dopamine-induced neurogenic vasodilatation, previously described in the isolated perfused hindleg of the dog, has been studied in anaesthetized dogs with intact circulation in the hindleg. Dopamine was administered intravenously and/or intra-aortically, either as a bolus injection of 4 or 16 μg/kg, or as a continuous infusion of 4, 8, 16 or 32 μg $kg^{-1}$ $min^{-1}$.

**2** Dopamine, given as a bolus injection or by infusion, reversibly inhibited synaptic transmission in the paravertebral lumbar ganglia, studied with preganglionic stimulation at 1 Hz. The inhibitory effect decreased gradually when the frequency of stimulation was increased to 16 Hz. The inhibition by dopamine was also present when spontaneous postganglionic activity was recorded. These effects were more pronounced on intra-aortic than on intravenous administration of dopamine.

**3** In about half of the animals studied, injection or infusion of dopamine elicited a decrease of vascular resistance in the innervated femoral artery, whereas systemic blood pressure either did not change or decreased. In the denervated femoral artery, an increase in vascular resistance was always observed.

**4** The decrease in femoral vascular resistance was considered to correspond with neurogenic vasodilatation caused by paravertebral ganglionic inhibition since (i) it only occurred in the innervated hindleg, (ii) blood pressure did not rise, (iii) this decrease was insensitive to atropine or propranolol and (iv) it was blocked by small doses of haloperidol. When hypovolemic shock was produced, the incidence of the neurogenic decrease of vascular resistance was smaller.

**5** Dopamine also increased renal blood flow. This increase was not reduced by the occurrence of the neurogenic vasodilatation in the innervated femoral artery.

**6** These results are consistent with the idea that the dopamine-induced neurogenic vasodilatation, originally described in the isolated perfused hindleg of the dog, also occurs when the circulation to the hindleg is intact. This suggests that, in the dog, the inhibitory effect of dopamine on sympathetic ganglia modulates its peripheral vasoconstrictor effects. In hypovolemic shock, where sympathetic nervous activity is high, the inhibitory effect of dopamine on sympathetic ganglia disappears and its direct vasoconstrictor effect on the vessels dominates.

## Introduction

In 1963 McDonald & Goldberg reported that a neurogenic vasodilatation occurs in the isolated perfused hindleg of the dog on systemic administration of dopamine. It has been suggested that this phenomenon, which is due to inhibition of sympathetic ganglionic transmission (Bogaert & De Schaepdryver, 1967; Willems, 1973; Willems & Bogaert, 1975a,b) is not involved in the systemic hypotensive effect of dopamine (Eble, 1964; Goldberg, 1972). It is not even known whether this neurogenic vasodilatation after systemic administration of dopamine takes place in the intact hindleg, as the direct effect of dopamine on the femoral vascular bed is a vasoconstrictor one (McNay, McDonald & Goldberg, 1965).

We therefore decided to study in detail the effect of intravenous administration of dopamine, as a single injection and by infusion, on the vascular resistance in the intact hindleg and on the ganglionic transmission in the abdominal lateral sympathetic chain of the dog. Dopamine was also given by the intra-aortic route, as earlier experiments were performed in this way (Willems, 1973; Willems & Bogaert, 1975a).

Preliminary results of this work have been published (Bogaert, Willems & De Schaepdryver, 1975).

## Methods

### *Ganglionic transmission*

Nineteen mongrel dogs of either sex, with body weights ranging between 4 and 8 kg, were used. The animals were anaesthetized with pentobarbitone sodium, in an intravenous dose of 30 mg/kg, further small doses being given as needed. The experimental preparation has been described in detail by Willems (1973). A bipolar platinum electrode was placed on the cut upper internodal segment of the fourth or fifth paravertebral ganglion and an electrical stimulus of 0,3 ms duration and supramaximal intensity was applied at a frequency of 1 Hz; in some experiments other frequencies were used, as indicated in the Results section. Postganglionic electrical activity was recorded by a second bipolar platinum electrode on the ramus communicans griseus of the ganglion. Permanent records were made on moving film with a Grass oscilloscope camera for simultaneous evaluation of the time course and the intensity of the effects of the substances studied. The results were measured as the maximal change in amplitude of the evoked spikes.

In 7 of the 19 experiments spontaneous electrical activity was followed on the oscilloscope. Recording from the undivided ramus gave reproducible electrical activity and allowed qualitative evaluation of the effect of dopamine.

### *Blood flow*

Twenty-nine mongrel dogs of either sex, with body weights ranging between 12 and 38 kg, were used. They were anaesthetized as described above. Mean blood flow was measured with a Statham Multiflo M-400 electro-magnetic flowmeter. Blood flow in the right innervated femoral artery was monitored in all 29 dogs. In 17 of these dogs, blood flow in the denervated left femoral artery was also measured. The left hindleg was denervated by cutting the sciatic nerve and the lumbar paravertebral chain on that side. In 8 of the 29 dogs, blood flow in the left renal artery was measured. Blood pressure was measured from the brachial artery with a Statham pressure transducer. By dividing mean blood pressure by mean flow (Statham Cardiovascular Analyzer SP 1011), vascular resistance in the right femoral bed was continuously obtained. Heart rate was derived from the ECG by a cardiotachometer coupler. Blood flows, arterial blood pressure, vascular resistance and heart rate were registered on a Beckman Dynograph recorder R. Changes in flow and resistance after a drug are expressed as a percentage of the values found immediately before the drug was given.

In some experiments, hypovolemic shock was produced by bleeding the animal from the carotid artery into a reservoir. Bleeding was stopped either at the moment vascular resistance in the innervated femoral artery rose by 30%, or at the moment the decrease in blood pressure attained a steady state.

### *Further methodological aspects*

Intravenous injections or infusions were given into the cephalic vein of the right foreleg. For intra-aortic injections or infusions, a catheter was introduced either via the left femoral artery (ganglionic transmission experiments), or via the left carotid artery (flow measurements) so that its tip was lying at the level of L4-L5.

Injections were always given in a volume of 0.2 ml, and 1 to 2 ml 0.9% w/v NaCl solution (saline) was used to flush the catheter. The agonists were injected in a single dose, the antagonists were injected slowly over 30 to 60 seconds. For control injections, saline was used, except for haloperidol, where the solvent of the latter was used. For the infusions, a volume of 0.5 ml/min, containing the appropriate concentration of the drug studied, was given during 5 to 10 minutes.

Values given in the text are means ± standard error of the mean. For statistical evaluation, Student's $t$-test was used.

### *Drugs*

The following drugs, diluted in saline, were used: dopamine hydrochloride (Sigma), (–)-noradrenaline bitartrate (Sigma), (±)-isoprenaline hydrochloride (Winthrop), haloperidol (Janssen Pharmaceutica), acetylcholine chloride (Roche), atropine sulphate (Boehringer-Sohn), propranolol hydrochloride (ICI), tetraethylammonium iodide (Merck). The solubilization of haloperidol by the use of lactic acid was described previously (Willems, 1973). Doses of the drugs are expressed as the base.

## Results

### *Ganglionic transmission*

*Preganglionic stimulation, 1 Hz, supramaximal intensity.* In 7 dogs dopamine (16 μg/kg) was injected intravenously; it produced an inhibition of the ganglionic transmission of 57.20 ± 6.29%. In 4 of these dogs the time course of the inhibition was followed: inhibition was maximal at 17.50 ± 1.04 s after injection. When the same dose of dopamine (16 μg/kg) was injected into the aorta, inhibition was more pronounced, 82.39 ± 4.74% ($n = 7$; $P < 0.05$) and occurred earlier, at 8.50 ± 0.55 s ($n = 4$; $P < 0.01$).

In the same series of dogs, dopamine was infused intravenously (8, 16 and 32 $\mu g\ kg^{-1}\ min^{-1}$) and intra-aortically (4, 8, and 16 $\mu g\ kg^{-1}\ min^{-1}$). For the duration of the infusion, a dose-dependent inhibition of the postsynaptic spike was observed; the results are given in Table 1A. As for single injections, the inhibitory effect on ganglionic transmission of infusions of dopamine is more pronounced for intra-aortic than for intravenous administration.

*Preganglionic stimulation, 1 Hz, intensity giving 50% of maximal response.* Stimulation was first performed with supramaximal intensity, at 1 Hz, and the doses of dopamine which produced a clear-cut, but not complete ganglionic inhibition, were determined. Stimulation was continued at 1 Hz, but stimulation intensity was diminished until the postsynaptic spike obtained was 50% of the spike obtained with maximal stimulation intensity. The same doses of dopamine were then again injected or infused. In these conditions, dopamine (16 µg/kg), injected intravenously in 5 dogs, produced an inhibition of ganglionic transmission of 68.60 ± 6.86%. This inhibition was larger ($P<0.05$) than that seen in the same dogs when stimulation intensity was maximal (48.80 ± 9.06%). The effect of lowering the intensity of stimulation, so that a 50% response was obtained, was also tested for infusions of dopamine; the results are summarized in Table 1B. In contrast to what happens with single injections, the effect of infusions of dopamine did not increase when a lower intensity of stimulation was used.

*Preganglionic stimulation, supramaximal intensity, varying frequencies.* In another series of 7 experiments, the intensity of stimulation was kept supramaximal but the frequency of stimulation was increased successively from 1 to 2, 4, 8 and 16 Hz, resulting in a decrease of the action potential amplitude of 14.21 ± 2.09% (2 Hz), 28.23 ± 4.74% (4 Hz), 34.92 ± 5.36% (8 Hz) and 50.57 ± 7.28% (16 Hz) respectively. At each frequency, once the amplitude was constant, dopamine was injected or infused in doses that produced near maximal inhibition at 1 Hz frequency of stimulation; this could only be obtained by giving dopamine intra-aortically. In 4 experiments, 8 µg/kg was injected. The ganglionic inhibition produced by this dose was of 71.81 ± 12.65% at 1 Hz, 69.84 ± 12.42% at 2 Hz, 65.08 ± 13.07% at 4 Hz, 43.69 ± 11.15% at 8 Hz and 12.25 ± 7.08% at 16 Hz: the inhibitory activity of dopamine decreases when the frequency of preganglionic stimulation is increased. Figure 1 shows a representative experiment.

In 3 experiments, 16 µg kg$^{-1}$ min$^{-1}$ was infused into the abdominal aorta. As for the single injections, the dopamine-induced ganglionic inhibition decreases with increasing frequency of stimulation: 86.67 ± 2.73% at 1 Hz, 60.67 ± 18.34% at 2 Hz, 55.33 ± 8.84% at 4 Hz, 26.00 ± 7.23% at 8 Hz and no inhibition at all at 16 Hz.

*Spontaneous activity.* Spontaneous electrical activity was recorded from the undivided postsynaptic nerve. Bursts of small potentials (10–20 µV) were observed at regular intervals (0.5–2 seconds). They completely disappeared upon injection of tetraethylammonium iodide (10 mg) into the abdominal aorta.

**Table 1** Inhibitory effect of infusions of dopamine on ganglionic transmission in the dog

A. *Supramaximal stimulation, 1 Hz*

| *Infusion* | *Dose (µg kg$^{-1}$ min$^{-1}$)* | | | |
|---|---|---|---|---|
| | *4* | *8* | *16* | *32* |
| i.v. | | 24.83<br>±2.85<br>*n*=6 | 35.90<br>±5.38<br>*n*=6 | 61.56<br>±7.40<br>*n*=5 |
| ao. | 36.00<br>±16.06<br>*n*=6 | 54.06<br>±10.48<br>*n*=7 | 75.38<br>±14.67<br>*n*=5 | |

B. *Stimulation giving 50% of maximal response, 1 Hz*

| | *Dose (µg kg$^{-1}$ min$^{-1}$)* | | |
|---|---|---|---|
| | *8* | *16* | *32* |
| i.v. | 20.73<br>±1.53<br>*n*=4 | 39.54<br>±5.20<br>*n*=5 | 59.70<br>±8.92<br>*n*=4 |
| ao. | 41.80<br>63.60<br>*n*=2 | 66.70<br>60.00<br>*n*=2 | |

i.v.=intravenous; ao.=intra-aortic. The inhibition is expressed as percentage of control spike, mean ± s.e. mean; when *n*=2, individual values are given.

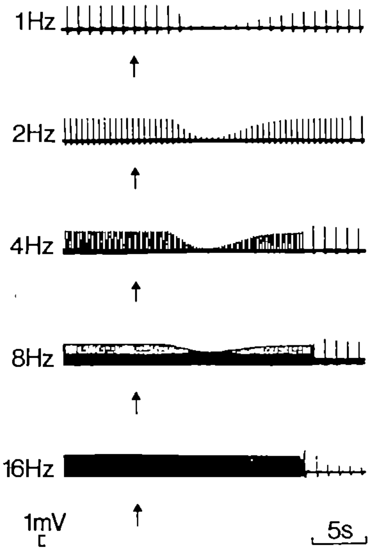

**Figure 1** Lumbar paravertebral ganglion of the dog. Recording electrode on the ramus communicans griseus, stimulating electrode on the upper internodal segment. Inhibitory effect of dopamine (at arrows), 8 µg/kg intra-aortically, at different stimulation frequencies.

In 5 experiments, dopamine was injected as a single dose (16 µg/kg) intravenously or intra-aortically; in all cases a short-lasting inhibition of the spontaneous electrical activity occurred with a time course similar to that observed in the stimulated preparation. No change in systemic blood pressure was observed except in 2 dogs in which intravenous dopamine provoked a blood pressure rise.

In 4 experiments, dopamine (8 and 16 µg $kg^{-1}$ $min^{-1}$) was infused intravenously, and in two of them also intra-aortically. Intravenous dopamine produced a decrease of the spike amplitude and spike frequency in 2 dogs; in one dog, blood pressure increased, whereas it decreased in the other. Intra-aortic dopamine decreased both spike amplitude and spike frequency; a blood pressure fall was seen in one dog.

### *Blood flow measurements*

*Denervated femoral artery.* Dopamine (4 and 16 µg/kg) was given as a bolus injection intravenously (13 preparations) and intra-aortically (16 preparations) in a series of 17 dogs; all responded with a fall of femoral blood flow; blood pressure either increased, decreased or did not change. In all dogs femoral resistance increased. These results are shown in Table 2, classified according to the change in systemic blood pressure seen.

(–)-Noradrenaline (0.1 µg/kg), injected intravenously or intra-aortically in some of these dogs, always caused an increase in systemic blood pressure and a decrease in flow (i.v.: +8.29 ± 1.66 mmHg; −25.11 ± 8.39%, respectively ($n=7$); ao.: +5.00 ± 1.00 mmHg; −62.47 ± 2.53% respectively ($n=4$)).

**Table 2** Effects of a bolus injection of dopamine on systemic blood pressure and on blood flow in the denervated femoral artery of the dog hindleg

| | *Intravenous injection* | | | |
|---|---|---|---|---|
| | *4 µg/kg (n=13)* | | *16 µg/kg (n=13)* | |
| *n* | 8 | 5 | 2 | 11 |
| BP | −16.00<br>±3.63 | +5.20<br>±0.80 | −23.00<br>−20.00 | ±11.45<br>±1.87 |
| F | −30.40<br>±4.92 | −40.68<br>±4.77 | −68.34<br>−57.70 | −66.47<br>±3.19 |
| | *Intra-aortic injection* | | | |
| | *4 µg/kg (n=10)* | | *16 µg/kg (n=6)* | |
| *n* | 5 | 5 | 2 | 4 |
| BP | −6.80<br>±2.80 | +4.00<br>±0.63 | −4.00<br>−12.00 | +6.50<br>±0.96 |
| F | −42.68<br>±6.11 | −54.39<br>±11.02 | −93.33<br>−79.17 | −86.10<br>±6.06 |

BP=Changes in blood pressure, mean ± s.e. mean in mmHg; F=percentage change in blood flow, mean ± s.e. mean. The experiments in which an increase in systemic blood pressure occurred are contrasted with the others; $n$ = number of animals showing the given combination of blood pressure change and blood flow change; for $n=2$, individual values are given.

In 13 of the 17 dogs, dopamine was infused intravenously or intra-aortically (4, 8 and 16 μg $kg^{-1}$ $min^{-1}$); it always elicited a decrease of femoral flow (Table 3). As with bolus injections, blood pressure either increased or decreased, but femoral resistance increased in all dogs. Intravenous infusion of (–)-noradrenaline (0.05, 0.1 or 0.2 μg $kg^{-1}$ $min^{-1}$) always increased systemic blood pressure and decreased femoral blood flow ($n = 7$).

*Innervated femoral artery.* When dopamine (4 and 16 μg/kg) was injected intravenously (in 25 and 26 respectively out of 29 dogs), or intra-aortically (in 13 and 8 respectively out of the same 29 dogs), different patterns of flow and blood pressure responses were observed. In Table 4 the results are classified according to the direction of the changes of blood pressure and femoral resistance. An increase in femoral vascular resistance (accompanying either an increase or a decrease in systemic blood pressure) occurred in less than half of the preparations with intact innervation. In the other dogs, femoral vascular resistance decreased; some of them showed an increase, others a decrease of systemic blood pressure. The fall of femoral vascular resistance in the dogs in which 4 μg/kg caused a decrease of systemic blood pressure, was most pronounced when dopamine was injected intra-aortically. Figure 2 shows a representative experiment, depicting femoral blood flow and systemic blood pressure.

(–)-Noradrenaline (0.1 μg/kg), injected intravenously in 11 dogs, led to a decrease in femoral resistance in 9 dogs (–35.02 ± 8.21%). In all animals

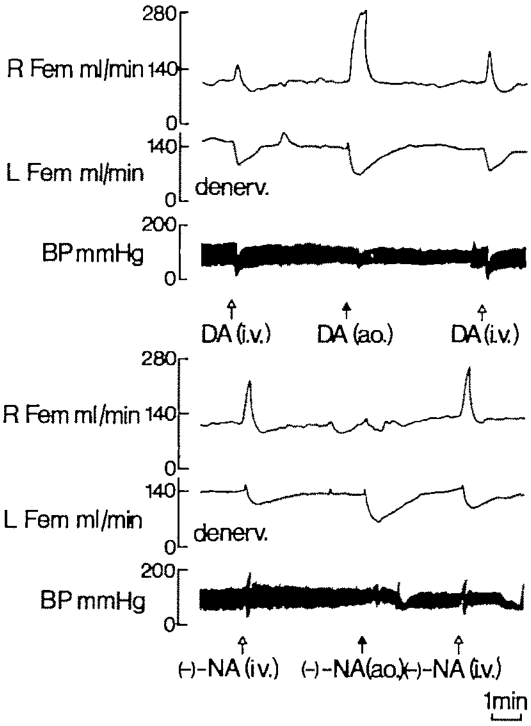

**Figure 2** Right (R Fem.) and left (L Fem.) femoral blood flow in the dog; the left hindleg has been denervated (denerv.); BP = systemic blood pressure. Dopamine (DA, 4 μg/kg) and (–)-noradrenaline ((–)-NA, 0.1 μg/kg) were injected intravenously (i.v.) or intra-aortically (ao).

**Table 3** Effects of a continuous infusion of dopamine on systemic blood pressure and on blood flow in the denervated femoral artery of the dog hindleg

| | *Intravenous Infusion* | | | | | |
|---|---|---|---|---|---|---|
| | *4 μg $kg^{-1}$ $min^{-1}$ (n=9)* | | *8 μg $kg^{-1}$ $min^{-1}$ (n=13)* | | *16 μg $kg^{-1}$ $min^{-1}$ (n=7)* | |
| *n* | 8 | 1 | 9 | 4 | 0 | 7 |
| BP | −3.00<br>±1.46 | +4.00 | −3.33<br>±2.16 | +12.00<br>±1.63 | | +14.29<br>±3.01 |
| F | −21.55<br>±1.62 | −44.40 | −37.57<br>±4.98 | −21.95<br>±4.01 | | −40.96<br>±7.09 |
| | *Intra-aortic infusion* | | | | | |
| | 4 μg $kg^{-1}$ *$min^{-1}$ (n=6)* | | *8* μg $kg^{-1}$ *$min^{-1}$ (n=3)* | | *16* μg $kg^{-1}$ *$min^{-1}$ (n=2)* | |
| *n* | 5 | 1 | 1 | 2 | 0 | 2 |
| BP | −2.00<br>±0.89 | +4.00 | −16.00 | +4.00<br>+4.00 | | +4.00<br>+8.00 |
| F | −55.06<br>±7.43 | −60.71 | −81.82 | −80.00<br>−66.67 | | −91.67<br>−82.61 |

BP = changes in blood pressure, mean ± s.e. mean in mmHg; F = percentage change in blood flow, mean ± s.e. mean. The experiments in which an increase in systemic blood pressure occurred are contrasted with the others; $n$ = number of animals showing the given combination of blood pressure change and blood flow change; for $n = 1$ or 2, individual values are given.

showing a decrease in resistance, an increase in systemic blood pressure (+11.33 ± 1.49 mmHg; $n=9$) occurred. In two dogs both blood pressure and femoral resistance increased. In 5 dogs the same dose of noradrenaline was also injected into the abdominal aorta; femoral resistance rose (+84.29 ± 32.06%) as did systemic blood pressure (+8.00 ± 3.10 mmHg).

Propranolol, given in a dose (0.3 mg/kg, i.v.), which blocked the vasodilator effect of isoprenaline (0.1 µg/kg, i.v.), had no effect on the decrease of femoral resistance elicited by dopamine (i.v. or ao.); it did not influence (–)-noradrenaline-induced reflex vasodilatation ($n=3$).

Atropine (1 mg/kg, i.v.; $n=1$) blocked the vasodilator response to acetylcholine (10 µg/kg, i.v.) without any effect on the decrease in femoral resistance elicited by dopamine (i.v.).

Haloperidol (0.1 mg/kg, i.v. or 1 mg, ao.; $n=6$) blocked the fall in femoral resistance in the innervated femoral artery by intravenous or intra-aortic dopamine without influencing the (–)-noradrenaline-induced reflex vasodilatation (Figure 3).

Tables 5 and 6 show the response patterns when dopamine was infused (4, 8 and 16 µg $kg^{-1}$ $min^{-1}$, i.v. and/or ao.). In these conditions, we observed a sustained decrease of femoral resistance in some dogs which showed a decrease of systemic blood pressure.

In 3 dogs, intravenous infusion of (–)-noradrenaline (0.05 µg $kg^{-1}$ $min^{-1}$) also produced a decrease of femoral resistance (–5.13 ± 5.13%) but this decrease was accompanied by an increase in systemic blood pressure (+7.33 ± 4.37 mmHg). Higher doses (0.1 and 0.2 µg $kg^{-1}$ $min^{-1}$) produced an increase in both femoral resistance and systemic blood pressure.

**Table 4** Effects of a single injection of dopamine on systemic blood pressure and on blood flow in the innervated femoral artery of the dog hindleg

| | *Intravenous injection* | | | |
|---|---|---|---|---|
| | *4 µg/kg* (n=*25*) | | *16 µg/kg* (n=*26*) | |
| *n* | 14 | 6 | 5 | 17 |
| BP | −13.71<br>±1.76 | +5.67<br>±0.80 | −19.80<br>±2.11 | +12.59<br>±1.28 |
| R | −32.00<br>±5.40 | −36.85<br>±11.01 | −60.93<br>±11.21 | −47.48<br>±7.06 |
| *n* | 3 | 2 | 0 | 4 |
| BP | −12.67<br>±7.51 | +2.00<br>+4.00 | | +13.00<br>±4.43 |
| R | +26.30<br>±13.30 | +13.60<br>+11.10 | | +63.39<br>±21.39 |
| | *Intra-aortic injection* | | | |
| | *4 µg/kg* (n=*13*) | | *16 µg/kg* (n=*8*) | |
| *n* | 5 | 2 | 3 | 2 |
| BP | −7.20<br>±2.65 | +4.00<br>+2.00 | −6.67<br>±4.81 | +8.00<br>+4.00 |
| R | −50.47<br>±13.14 | −45.45<br>−16.67 | −49.61<br>±10.87 | −80.00<br>−50.00 |
| *n* | 1 | 5 | 1 | 2 |
| BP | −2.00 | +4.40<br>±0.40 | −12.00 | +6.00<br>+8.00 |
| R | +83.83 | +57.97<br>±18.96 | +73.33 | +211.11<br>+66.67 |

BP=changes in blood pressure, mean ± s.e. mean in mmHg; R=percentage change in vascular resistance, mean ± s.e. mean. The experiments are classified in four groups: no increase in blood pressure and femoral resistance, no increase in blood pressure with increase in femoral resistance, increase in blood pressure and femoral resistance, increase in blood pressure with no increase in femoral resistance; *n*=number of animals showing the given combination of blood pressure change and change in femoral resistance; for *n*=1 or 2, individual values are given.

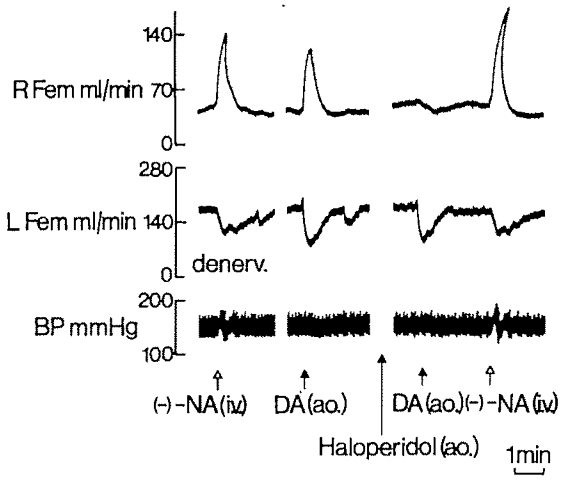

**Figure 3** Right (R Fem.) and left (L Fem.) femoral blood flow in the dog; the left hindleg has been denervated (denerv.); BP=systemic blood pressure. Dopamine (DA, 2 μg/kg intra-aortically), (–)-noradrenaline ((–)-NA, 0.1 μg/kg intravenously), haloperidol (1 mg intra-aortically).

*Renal artery and femoral artery blood flow.* Dopamine (16 μg/kg, injected i.v.; $n=6$) increased both renal artery blood flow (+13.88±3.28%) and blood pressure (+16.00±2.31 mmHg).

Intravenous infusion of 4 μg $kg^{-1}$ $min^{-1}$ of dopamine ($n=7$) caused in 5 dogs a decrease of systemic blood pressure (–2.40±1.60 mmHg) and an increase in renal flow (+19.30±4.89%). In 2 dogs a decrease of both blood pressure and renal blood flow occurred. Intravenous infusion of 8 μg $kg^{-1}$ $min^{-1}$ of dopamine ($n=7$), elicited in 4 dogs a decrease in systemic blood pressure (–9.00±3.42 mmHg) and an increase in renal flow (+27.75±5.02%). In 2 dogs blood pressure and renal flow rose. In one dog no changes in blood pressure and renal flow were observed with this dose of dopamine. Intravenous infusion of 16 μg $kg^{-1}$ $min^{-1}$ of dopamine ($n=4$) produced an increase in blood pressure (+18.00±1.15 mmHg) and an increase in renal flow (+16.40±3.61%). In 5 of the dogs that showed an increase in renal blood flow, the infusion of dopamine caused a decrease in femoral resistance: this decrease started after the renal dilatation was already present. In none of the dogs was the occurrence of the femoral dilatation accompanied by a decrease in renal flow.

*Femoral artery blood flow and severe blood loss.* In 8 dogs, blood was withdrawn from the left femoral artery: in 5 dogs until vascular resistance in the intact right femoral bed increased by 30 to 50% and in 3 dogs bleeding was continued until systemic blood pressure stabilized at a lower level. When dopamine was then infused intravenously (8 μg $kg^{-1}$ $min^{-1}$) only 1 out of the 8 dogs showed a decrease of both femoral resistance (–8.30%) and systemic blood pressure (–4.00 mmHg). In 2 other dogs a decrease in femoral resistance was accompanied by an increase in blood pressure. In 5 dogs both femoral resistance and systemic blood pressure increased.

**Table 5** Effects of an intravenous infusion of dopamine on systemic blood pressure and on blood flow in the innervated femoral artery of the dog hindleg

| | *4 μg $kg^{-1}$ $min^{-1}$ (n=20)* | | *8 μg $kg^{-1}$ $min^{-1}$ (n=25)* | | *16 μg $kg^{-1}$ $min^{-1}$ (n=14)* | |
|---|---|---|---|---|---|---|
| *n* | 7 | 2 | 6 | 7 | 1 | 7 |
| BP | −2.57<br>±1.29 | +4.00<br>+4.00 | −2.00<br>±1.37 | +7.43<br>±1.84 | −32.00 | +18.86<br>±1.90 |
| R | −27.88<br>±7.99 | 0<br>0 | −28.77<br>±5.72 | −29.02<br>±6.85 | −38.46 | −46.31<br>±8.47 |
| *n* | 7 | 4 | 10 | 2 | 1 | 5 |
| BP | −5.43<br>±1.43 | 0 | −6.20<br>±2.28 | +12.00<br>+4.00 | −4.00 | +12.00<br>±2.83 |
| R | +16.36<br>±4.81 | 0 | +19.52<br>±8.08 | +23.50<br>0 | +29.17 | +45.02<br>±34.45 |

BP=changes in blood pressure, mean±s.e. mean in mmHg; R=percentage change in vascular resistance, mean±s.e. mean. The experiments were classified in four groups: no increase in blood pressure and femoral resistance, no increase in blood pressure with increase in femoral resistance, increase in blood pressure and femoral resistance, increase in blood pressure with no increase in femoral resistance; $n$=number of animals showing the given combination of changes in blood pressure and in vascular resistance; for $n=1$ or 2, individual values are given.

## Discussion

This study was performed in order to investigate in the intact hindleg of the dog the dopamine-induced neurogenic vasodilatation which had hitherto only been studied in the dog's isolated perfused hindleg (McDonald & Goldberg, 1963; Bogaert & De Schaepdryver, 1967; Willems & Bogaert, 1975a). The doses and the infusion rates of dopamine, used in the experiments presented here, were chosen because they show the experimentally and clinically interesting effects of dopamine: renal vasodilatation, increase in cardiac contractility, decrease in peripheral resistance, slight or no decline of systemic blood pressure and absence of chronotropic effect (McDonald & Goldberg, 1963; Goldberg, 1972; Ramdohr, Schüren, Biamino & Schröder, 1973; Setler, Pendleton & Finlay, 1975).

In many of the dogs, systemic administration of dopamine decreased femoral vascular resistance. However, only those experiments in which a fall in systemic blood pressure accompanies the decrease in femoral resistance, can be used to demonstrate a neurogenic mechanism because of the ganglionic inhibitory effect of dopamine (Willems, 1973). Indeed, if a decrease in femoral resistance occurs with increased systemic blood pressure, a neurogenic vasodilatation cannot be distinguished from reflex baroreceptor-induced changes in vascular resistance. Dopamine, injected intravenously, in some animals did produce a decrease of femoral resistance without concomitant increase in systemic blood pressure: this was observed in 14 out of 25 dogs receiving 4 µg/kg of dopamine and in 5 out of 26 dogs receiving 16 µg/kg.

The following observations suggest that the femoral dilatation, occurring without increase in blood pressure, corresponds to the neurogenic vasodilatation described in the isolated perfused hindleg.

A decrease in femoral vascular resistance after systemic administration of dopamine was only observed in the innervated hindleg; in the denervated hindleg an increase in vascular resistance always occurred. This vasoconstriction is due to a direct effect of dopamine on the femoral vasculature where it interacts with the α-adrenoceptor or with the 5-hydroxytryptamine receptor (Gilbert & Goldberg, 1975). Vasoconstriction also occurs when dopamine is injected directly into the innervated femoral artery (McDonald & Goldberg, 1963; McNay *et al.*, 1965). It is only after the administration of α-receptor antagonist drugs that the direct effect of dopamine on the femoral artery is a vasodilatation (McNay & Goldberg, 1966; Higgins, Millard, Braunwald & Vatner, 1973; Bell, Conway, Lang & Padanyi, 1975).

A fall in vascular resistance, requiring intact innervation of the hindleg, was also observed after the intravenous administration of (–)-noradrenaline, but in this case the fall in resistance was always accompanied by an increase in systemic blood pressure, suggesting that a classical baroreceptor-reflex is involved. Intra-aortic administration of (–)-noradrenaline always caused an increase in femoral resistance. We have shown previously that the doses of (–)-noradrenaline and dopamine needed for ganglionic inhibition are the same (Willems, 1973); this is also true for the neurogenic vasodilatation (Willems & Bogaert, 1975a). Such doses of (–)-noradrenaline are too high to be used in these experiments because they always give a pronounced increase in systemic pressure.

Our results confirm our previous reports (Willems, 1973; Willems & Bogaert, 1975a) that dopamine,

**Table 6** Effects of an intra-aortic infusion of dopamine on systemic blood pressure and on blood flow in the innervated femoral artery of the dog hindleg.

| | *4 µg kg⁻¹ min⁻¹ (n=7)* | | *8 µg kg⁻¹ min⁻¹ (n=4)* | | *16 µg kg⁻¹ min⁻¹ (n=4)* | |
|---|---|---|---|---|---|---|
| *n* | 0 | 1 | 0 | 1 | 0 | 0 |
| BP | | +4.00 | | +4.00 | | |
| R | | −41.38 | | −66.67 | | |
| *n* | 5 | 1 | 2 | 1 | 1 | 3 |
| BP | −2.40<br>+0.75 | +4.00 | −16.00<br>−2.00 | +4.00 | −4.00 | +6.67<br>±1.33 |
| R | +29.77<br>±8.06 | +30.77 | +21.43<br>0 | +133.33 | +52.38 | +134.11<br>±75.86 |

BP=changes in blood pressure, mean±s.e. mean in mmHg; R=percentage change in vascular resistance, mean±s.e. mean. The experiments were classified in four groups: no increase in blood pressure and femoral resistance, no increase in blood pressure with increase in femoral resistance, increase in blood pressure and femoral resistance, increase in blood pressure with no increase in femoral resistance; *n*=number of animals showing the given combination of blood pressure change and change in vascular resistance; for *n*=1 or 2, individual values are given.

within the dose range used here, inhibits ganglionic transmission in the paravertebral ganglion when injected intra-aortically. Moreover they show that the same effect occurs after intravenous administration. The experiments using sub-maximal preganglionic stimulation, which is closer to the physiological situation than supramaximal stimulation, and the experiments with recording of spontaneous sympathetic activity demonstrate that the same phenomenon probably also occurs in the normally activated ganglion.

The fact that the ganglionic inhibition is less pronounced when a dose of dopamine is injected intravenously than intra-aortically parallels the observation by Bogaert & De Schaepdryver (1967) that for the same dose of dopamine, the neurogenic vasodilatation in the isolated perfused preparation is larger when it is injected intra-aortically. This is also suggested by the fact that in those experiments, in which 4 µg/kg of dopamine decreased both resistance in the innervated hindleg and systemic blood pressure, the effect on the femoral flow is larger when dopamine is injected intra-aortically than intravenously. That the same phenomenon does not occur with 16 µg/kg is probably due to the more pronounced direct vasoconstriction when that dose is given.

Propranolol and atropine, in doses antagonizing $\beta$-mimetic and muscarinic vasodilatation respectively, did not influence the vasodilatation elicited by intravenous dopamine. Haloperidol, which selectively inhibits dopamine-induced ganglionic inhibition (Willems, 1973) and dopamine-induced neurogenic vasodilatation in the isolated perfused hindleg (Willems & Bogaert, 1975a), blocked the dopamine-induced decrease in femoral resistance without any influence on the reflex dilatation produced by (−)-noradrenaline. The direct vasodilator effect of dopamine in the femoral vascular bed, which is observed after $\alpha$-adrenoceptor blockade, has been attributed to $\beta$-adrenoceptor stimulation (McNay & Goldberg, 1966) but others have found this effect to be resistant to $\beta$-receptor blockade (Higgins *et al.*, 1973), and antagonized by ergometrine (Bell *et al.*, 1975). In our experiments, no $\alpha$-receptor antagonist was given and intra-femoral dopamine elicited only a vasoconstriction. It is therefore unlikely that the fall of resistance which we observed in our experiments and which was found to be resistant to $\beta$-blockade but sensitive to haloperidol, can be explained by a direct effect on the femoral vasculature. In our infusion experiments, dopamine also inhibited ganglionic transmission for the duration of the infusion. Therefore in those experiments, in which vascular resistance decreased in the innervated hindleg during a continuous dopamine-infusion without increase in systemic blood pressure, we can assume that a neurogenic vasodilatation, caused by ganglionic inhibition, is present.

In our experiments, a small increase in renal blood flow occurred, which is in agreement with earlier observations (see Goldberg, 1972; Setler *et al.*, 1975). The neurogenic fall of femoral resistance, which we observed, did not influence the renal vasodilatation, the increase in femoral blood flow being probably too small to decrease renal blood flow.

Only 1 out of 8 dogs with reduced blood volume showed neurogenic vasodilatation with 8 µg $kg^{-1}$ $min^{-1}$ of dopamine, in contrast to 6 out of 25 normal animals. This is perhaps due to the decreasing ability of dopamine to inhibit ganglionic transmission in that situation; this is suggested by our observation that dopamine is in fact less potent as an inhibitor of transmission when stimulus frequency in the ganglionic preparation is increased, thus mimicking increased sympathetic activity in shock (Folkow, 1952). Very recently it has been suggested (Hall, Schwinghamer & Lalone, 1976) that after a rapid haemorrhage the increased vascular resistance in a dog limb is mainly due to circulating catecholamines and not to increased sympathetic tone. This could be an alternative explanation.

Finally we would conclude that, although the neurogenic vasodilatation induced by dopamine is not essential for its hypotensive effect (Eble, 1964; Goldberg, 1972), it is present in some animals and, as such, could contribute to the systemic effect.

*Note added in proof:* After completion of this paper, we observed that occasionally dogs respond to *local, intra-femoral injection* of dopamine in the innervated hindleg with a biphasic response: vasoconstriction followed by vasodilatation, a response similar to that described by Higgins *et al.* (1973). This phenomenon is clearly different from the response *to intravenous or intra-aortic administration* of dopamine described in our paper, i.e. vasodilatation preceding and dominating an occasional vasoconstriction (Figure 2).

This work was aided by grants from the Coordination Committee for Scientific Research (C.C.A.S.) and from the Fund for Interdisciplinary Research, Belgium.

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*(Received June 14, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 293–301

# THE EFFECTS OF INTRAPORTAL INJECTIONS OF NORADRENALINE, ADRENALINE, VASOPRESSIN AND ANGIOTENSIN ON THE HEPATIC PORTAL VASCULAR BED OF THE DOG: MARKED TACHYPHYLAXIS TO ANGIOTENSIN

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**1** The hepatic portal vein of the anaesthetized dog was cannulated and perfused with blood derived from the cannulated superior mesenteric vein.

**2** The portal vein was perfused at constant flow, the hepatic portal venous pressure being monitored continuously together with the inferior vena caval pressure. From these measurements, the hepatic portal venous vascular resistance was calculated.

**3** Noradrenaline and adrenaline were injected intraportally in graded doses which caused dose-dependent increases in the hepatic portal vascular resistance. At all doses, adrenaline was significantly ($P<0.05$) more potent than noradrenaline.

**4** Intraportal injections of vasopressin caused reductions in calculated hepatic portal venous vascular resistance in most experiments; these effects were dose-dependent.

**5** No tachyphylaxis to the effects of noradrenaline, adrenaline or vasopressin was observed.

**6** Intraportal injections of angiotensin caused dose-dependent increases in calculated hepatic portal vascular resistance up to 5 μg; thereafter larger doses caused smaller increases in portal resistance.

**7** Repeated intraportal injections of angiotensin revealed the existence of tachyphylaxis in the hepatic portal vascular bed.

**8** Intraportal infusions of angiotensin caused rises in calculated hepatic portal vascular resistance from which there was almost complete 'escape' despite the continued infusions. Infusions of noradrenaline which caused similar rises in calculated portal vascular resistance did not exhibit equivalent degrees of 'escape'.

**9** The development of tachyphylaxis explains the fact that doses of 10 and 20 μg of angiotensin injected after 5 μg doses produced smaller effects. If a much longer time interval was allowed between injections (30 min), the dose-response curve to angiotensin had a sigmoid shape.

**10** These findings are discussed with respect to their possible importance in the functional status of the hepatic portal vascular bed in this species.

## Introduction

Injections of noradrenaline, angiotensin and vasopressin into the hepatic artery of the dog have been shown to cause constriction of the hepatic arterial vascular bed (Richardson & Withrington, 1976a). Similarly, adrenaline, at even the lowest doses appears to be vasoconstrictor to the denervated hepatic arterial vascular tree (Andrews, Hecker, MacGraith & Ritchie, 1955; Richardson & Withrington, unpublished observations). In addition to the arterial inflow, the liver receives a large supply of blood which has drained the intestine, pancreas and spleen via the portal vein. Varying amounts of noradrenaline, adrenaline, vasopressin and angiotensin may therefore enter the liver in the portal vein, having passed through the mesenteric, pancreatic and splenic vasculature. These vasoactive agents may affect the portal vascular circuit by actions on structures within the liver which control portal vascular resistance. The present paper describes the actions of these naturally-occurring substances on the portal vascular resistance studied by the use of a perfusion technique where the blood was derived from the mesenteric vein. The portal blood flow was maintained constant despite alterations in the mesenteric and other series-coupled vascular territories which, normally, would alter portal haemodynamics.

The actions of angiotensin were examined in more detail than those of the other substances because our experiments revealed that successive intraportal injections and infusions resulted in tachyphylaxis, a phenomenon seen neither with the other agents nor in studies of angiotensin on the hepatic arterial vascular bed.

## Methods

Experiments were performed on 7 dogs weighing between 9.6 and 20.6 kg (15.7 ± 3.5 kg: mean ± s.d.) which had been starved, but allowed free access to water for 24 h before the induction of anaesthesia with methohexitone sodium (Brietal, Lilly, 7.5–10.0 mg/kg, i.v.). Anaesthesia was maintained with intravenous chloralose (Kuhlmann, Paris: 50 mg/kg) and urethane (BDH; 500 mg/kg), supplements of chloralose and urethane being given as necessary to maintain a constant level of anaesthesia.

### *Surgical preparation*

Following a midline laparotomy, the splenic, superior mesenteric and hepatic portal veins were dissected free of surrounding tissue. The splenic artery and its accompanying periarterial sympathetic nerves were then dissected free, tied off, and the nerves stimulated supramaximally to expel the erythrocytes stored within the spleen into the systemic circulation. After an intravenous injection of heparin (Weddel Pharmaceuticals: 250 iu/kg and hourly supplements of 100 iu/kg) to prevent blood coagulation, the splenic vein was tied. The vessel was then cannulated towards the liver with wide bore tubing, and connected to a small reservoir into which the effluent from the superior mesenteric vein subsequently drained. The hepatic portal vein was tied about 5 mm from the confluence of the splenic and superior mesenteric veins, and the superior mesenteric venous effluent drained via the cannulated splenic vein into the reservoir. The spleen was then removed.

The superior mesenteric venous blood draining into the reservoir was returned to the animal via a cannulated external jugular vein until the hepatic portal vein perfusion was established.

The hepatic portal vein was then cannulated towards the liver with wide-bore tubing, and the hepatic portal venous vascular bed perfused at constant flow with a Watson-Marlow MHRE200 roller pump deriving its supply from the reservoir receiving blood from the superior mesenteric vein. The pump system was set to perfuse the hepatic portal vein at the same flow as that draining from the superior mesenteric vein via the cannulated splenic vein into the reservoir (see Results section). Interposed between the roller pump system and the hepatic portal venous cannula were a cannulating flowhead, 'T'-pieces for the injection and infusion of vasoactive substances into the hepatic portal vein, and a further 'T'-piece close to the point of cannulation of the hepatic portal vein for the measurement of the hepatic portal venous perfusion pressure.

Once perfusion of the hepatic portal venous vascular bed had been established, the laparotomy incision was closed and a thermometer inserted into the abdominal cavity; the intra-abdominal temperature was maintained at 37–38°C throughout all experiments, by the use of radiant lamps and table heaters. The volume contained in the external perfusion circuit was compensated for with a solution of 10% low molecular weight dextran in normal saline (Rheomacrodex, Pharmacia).

### *Recording of variables*

*Systemic arterial blood pressure (BP)* was measured from a cannulated femoral artery with a Statham P23Gb strain gauge transducer; pulsatile pressure was recorded throughout all experiments.

*Heart rate (HR)* was measured with a Devices 4521 ratemeter triggered from the pulsatile systemic arterial pressure waveform.

*Inferior vena cava pressure (IVCP)* was measured from a catheter passed into the inferior vena cava via a femoral vein for a distance estimated to cause its tip to lie close to the entrance of the hepatic vein into the inferior vena cava; the position of the catheter tip was confirmed *post mortem*. The catheter was connected to a Consolidated Electrodynamics L212 strain gauge transducer, and the mean IVCP derived with a Devices 3502 averaging circuit with time constants of 0.5, 1 or 2 s selected appropriately.

*Hepatic portal venous blood flow (HPVF)* was monitored with a cannulating flowhead and electromagnetic flowmeter (Cardiovascular Instruments, Model 3765T), with the flowhead on the outflow side of the roller pump used for perfusing the hepatic portal venous vasculature. Hydrostatic flow zeroes were established throughout each experiment by diverting the blood through a bypass in parallel with the flowprobe; this avoided interruption of the blood flow whilst establishing zero positions. The flow measuring system was calibrated at the end of each experiment *in situ*, with whole blood. Mean blood flow was obtained by passing the phasic waveform through an averaging circuit with a time constant of 0.6 s; both mean and phasic signals were recorded continuously.

*Hepatic portal venous pressure (HPVP)* was measured from a 'T'-piece in the cannula just before the point of cannulation of the hepatic portal vein, using a Statham P23V strain-gauge transducer. Mean hepatic portal venous pressure was obtained by passing the phasic signal through a Devices 3502 averaging circuit with a time constant of 0.5, 1.0 or 2.0 s selected appropriately.

*Superior mesenteric venous outflow (SMVF)* was monitored with an electromagnetic flowmeter (Cardiovascular Instruments C500) and a cannulating flowprobe in the cannula draining the splenic vein. Flow zeroes and calibrations were obtained as described above.

All pressure transducers were precalibrated with mercury or water manometers: zero reference positions were checked frequently throughout the course of each experiment.

After suitable amplification, all variables were recorded continuously on a Devices M19 rectilinear recorder.

### *Calculations*

*Liver weight.* The livers were excised and weighed immediately *post mortem*; values expressed per 100 g refer to this terminal weight of liver.

*Hepatic portal venous vascular resistance (HPVR).* The hepatic portal venous inflow remained constant at all times in each experiment, as did the inferior vena caval pressure. Thus an increase and decrease in HPVP indicated a similar directional change in HPVR. Vascular resistance was calculated as (hepatic portal mean perfusion pressure–inferior vena cava pressure)/(hepatic portal mean blood flow) and expressed as mmHg ml$^{-1}$ min, or mmHg ml$^{-1}$ min 100 g. Changes in HPVR were calculated as the percentage change in vascular resistance from control values immediately before a drug injection to the peak of the response, i.e., (change in HPVR x 100)/(control HPVR).

### *Expression of results*

Except where indicated to the contrary, results are expressed as means ± s.e. means. Degrees of statistical significance were assessed by Student's *t*-test for paired data samples.

Log$_{10}$ dose-response curves were constructed by injecting increasing, graded, doses of vasoactive substances into the hepatic portal vein, the doses being increased either until the maximum response was attained, or until pronounced systemic effects resulted from the drugs passing through the liver and entering the systemic circulation. Unless stated to the contrary in the results, one dose of a drug was injected about 1 min after the complete recovery from the effects of the previous injection.

### *Vasoactive substances*

The following drugs were used in this investigation: adrenaline bitartrate (Macarthays), noradrenaline acid tartrate (Levophed, Winthrop), angiotensin II amide (Hypertensin, Ciba), vasopressin (Pitressin for i.v. injection, Parke-Davis). Doses are expressed in terms of the weight of salt injected (for noradrenaline, the weight of base injected) and for vasopressin in international units (iu) of activity where 1 unit is equivalent to 0.5 mg (manufacturer's data).

Drugs were injected into the cannula between the roller pump and the hepatic portal vein (intraportally) in volumes between 0.5 and 1.0 ml, washed in with 0.9% w/v NaCl solution (saline) to a total injectate volume of 2.0 ml; these injections resulted in small injection artifacts which were clearly separable from the subsequent drug-induced effects.

Infusions were made into the portal vein cannula (intraportally) from a Watson-Marlow MHRE200 pump precalibrated to deliver 1.0 ml/min against the range of pressures encountered in these experiments. All infusions were of 5 min duration.

## Results

### *Control values*

In the present experiments, the livers weighed 307.1 ± 55.8 (mean ± s.d.) grams. In each experiment when the hepatic portal venous perfusion was established such that the portal blood flow was equal to the superior mesenteric venous outflow (see Methods section), the following values for the control variables (mean ± s.d.) were obtained: systemic arterial mean pressure (BP) 140.7 ± 13.7 mmHg, heart rate 157.1 ± 38.0 beats/min and hepatic portal venous blood flow 240.7 ± 62.7 ml/min or 78.5 ± 14.3 ml min$^{-1}$ 100 g$^{-1}$ with a portal mean perfusion pressure of 6.6 ± 3.2 mmHg. Under these conditions, the mean inferior vena cava pressure was 1.8 ± 0.8 mmHg. The mean value for the HPVR was 0.066 ± 0.035 mmHg ml$^{-1}$ min 100 g, or hepatic portal vascular conductance of 20.1 ± 12.8 ml min$^{-1}$ 100 g$^{-1}$ mmHg$^{-1}$.

The values for the systemic arterial pressure and heart rate are similar to those reported previously in the anaesthetized dog whilst the mean portal vascular resistance is considerably less than the calculated hepatic arterial vascular resistance in either the sympathetically-denervated (Richardson & Withrington, 1976a) or sympathetically-innervated (Richardson & Withrington, 1976b,c,d) liver preparations reported previously from this laboratory.

### *Effects of intraportal injections of vasoactive substances on the hepatic portal vascular resistance*

In each experiment, the intraportal injection of saline (0.5 or 1.0 ml) washed in, in the same way as for a

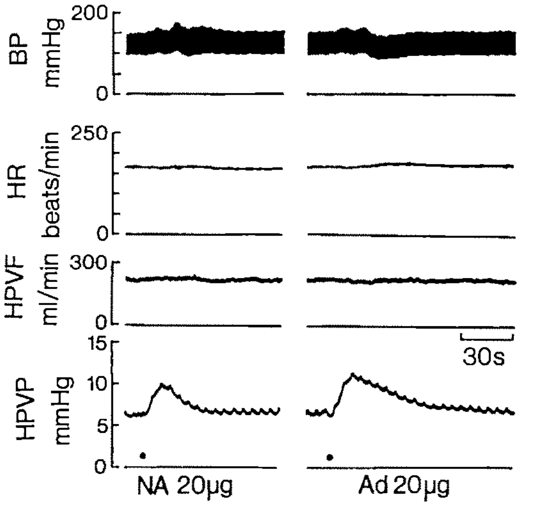

**Figure 1** Effects of intraportal injections of noradrenaline (NA) 20 μg, and adrenaline (Ad) 20 μg, on the canine hepatic portal venous vascular bed perfused at constant inflow. BP = systemic arterial blood pressure, HR = heart rate, HPVF = mean hepatic portal venous blood flow, and HPVP = mean hepatic portal venous perfusion pressure. Dots indicate the points of intraportal injections.

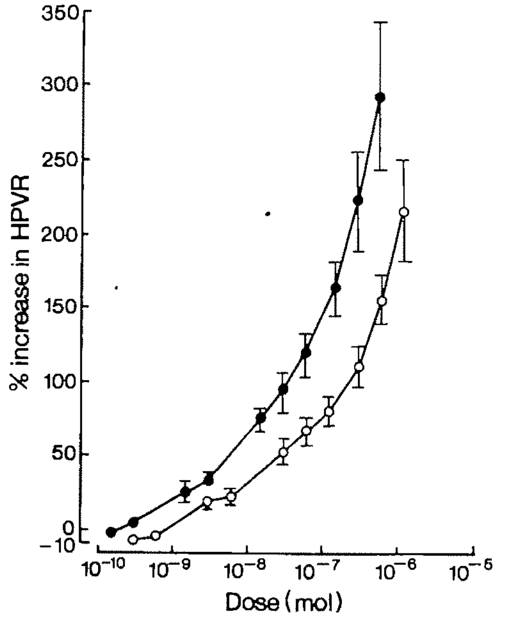

**Figure 2** $\text{Log}_{10}$ dose-response curves for the increase in hepatic portal vascular resistance (HPVR) to the intraportal injection of adrenaline (●) and noradrenaline (○). Abscissa scale: dose of drug in moles; ordinate scale: percentage increase in hepatic portal vascular resistance. Points represent the mean of 5 determinations; vertical bars show the s.e. means.

drug injection was without measurable effect on the HPVR.

*Noradrenaline.* In 6 preparations, the $\log_{10}$ dose-response relationship between the intraportal injection of increasing graded doses of noradrenaline, and the resulting change in the calculated HPVR was established on 7 occasions. Noradrenaline was injected in selected doses over the range 0.05 to 200 μg in each experiment; intraportal doses of noradrenaline in excess of 10 μg were accompanied by changes in systemic arterial pressure and heart rate. The peak changes in hepatic portal venous perfusion pressure which were used to calculate changes in HPVR (see Methods section) were not temporally related to these systemic effects (Figure 1), and usually preceded them by 10–15 seconds.

Doses in excess of 200 μg were not administered since there were marked systemic cardiovascular effects arising from the noradrenaline passing through the liver into the systemic circulation. The responses of the portal venous vascular bed to the largest doses of noradrenaline used in this study might not, therefore, have been the absolute maximum attainable in other types of preparation.

Of the 7 injections of noradrenaline at or below 0.1 μg, 4 evoked a reduction in hepatic portal venous pressure indicating a reduction in calculated HPVR. The other 3 injections did not cause any measurable change in the calculated portal vascular resistance.

All injections above 0.1 μg evoked increases in HPVP (Figure 1) representing increases in calculated HPVR. The rise in HPVR was graded and increased with increasing intraportal injections of noradrenaline (Figure 2).

There was no evidence in any experiment of tachyphylaxis to repeated intraportal injections of noradrenaline, which were usually made within 1 min of the hepatic portal perfusion pressure returning to the pre-injection baseline level.

*Adrenaline.* The $\log_{10}$ dose-response relationship between intraportal injections of adrenaline and the changes in HPVR were established over the same nominal weight range as for noradrenaline, 0.05 to 200 μg, in each of five preparations. In all of these experiments, the injections of adrenaline were paired with intraportal injections of equal weights of noradrenaline to compare the relative potencies of the two catecholamines.

On 2 out of 6 occasions in which adrenaline was injected in a dose of 0.1 μg or less, there was a reduction in hepatic portal perfusion pressure representing a reduction in the calculated HPVR.

All intraportal injections in excess of 0.1 μg adrenaline evoked increases in the hepatic portal perfusion pressure and rises in the calculated HPVR. This increase in HPVR in response to adrenaline increased with the dose injected intraportally; doses of adrenaline in excess of 10 μg were accompanied by changes in systemic arterial pressure and rises in heart rate. As with noradrenaline, the peak increase in hepatic portal venous perfusion pressure preceded the peak systemic changes by 10–15 s (Figure 1). Doses in excess of 200 μg were not administered since substantial systemic effects resulted from the intraportal injection of this dose of adrenaline. There was no evidence in any experiment of hepatic portal vascular tachyphylaxis to repeated intraportal injections of adrenaline.

In all five experiments in which paired doses of noradrenaline and adrenaline were injected into the hepatic portal vascular bed, it was evident that at each dose, adrenaline was more potent than noradrenaline in producing increases in hepatic portal perfusion pressure, and consequently in the calculated HPVR. The difference between the response to noradrenaline and adrenaline was statistically significant when paired doses of 1, 5, 10, 20, 50, 100 and 200 μg were injected intraportally ($P < 0.01$, 0.02, 0.05, 0.01, 0.01, 0.05 and 0.05 respectively). These weights of noradrenaline are expressed in terms of the base, and those of adrenaline as the bitartrate salt; if, however, the doses are expressed on a molar basis, the difference in potency between these two catecholamines becomes even more apparent (Figure 2).

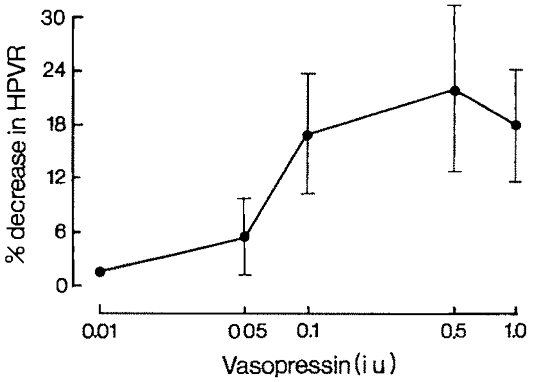

**Figure 3** Dose-response curve for the reduction in hepatic portal vascular resistance (HPVR) to intraportal administrations of vasopressin. Abscissa scale: $\log_{10}$ dose of drug in international units (iu); ordinate scale: percentage decrease in hepatic portal vascular resistance. Points represent the means of 5 experiments, vertical bars show the s.e. means.

*Vasopressin* was injected intraportally on 24 occasions in 6 experiments in doses from 0.005 to 5.0 iu. On 18/24 occasions, there was a small, slightly delayed reduction in the hepatic portal perfusion pressure and therefore in the calculated hepatic portal vascular resistance. On the remaining 6/24 injections, not restricted to low doses of vasopressin, no change in hepatic portal perfusion pressure was observed subsequent to the intraportal injection of vasopressin. Nevertheless, the mean results from 6 experiments indicate that the reduction in calculated HPVR in response to intraportal vasopressin was graded and dose-dependent (Figure 3). On no occasion in any experiment was intraportal administration of any dose of vasopressin accompanied by an increase in calculated HPVR.

In most experiments, the higher doses of vasopressin administered intraportally evoked a reduction in calculated HPVR and subsequently, after passage through the cardiopulmonary circuit into the systemic arterial system, caused mesenteric vasoconstriction reflected by a reduction in the outflow from the superior mesenteric vein (see Methods section) with either no change or a concomitant rise in the systemic arterial blood pressure.

*Angiotensin.* The complete $\log_{10}$ dose-response relationship for angiotensin on the hepatic portal venous vascular bed was established in each of 6 experiments by graded, increasing, intraportal injections of between 0.05 and 20 μg. The doses were usually given within 1 min of the hepatic portal perfusion pressure returning to the control level, an interval between injections of approximately 5 minutes. In all experiments, all doses of angiotensin administered in this way caused an increase in the hepatic portal perfusion pressure and in the calculated HPVR. This increase in portal resistance was graded and dose-dependent (Figure 4). However, in all experiments, with the injection procedure described above, the magnitude of the increases in HPVR increased up to doses of 5.0 μg intraportally, but higher doses (10 and 20 μg) resulted in smaller increases in the calculated hepatic portal venous vascular resistance than were elicited by 5.0 μg, and a bell-shaped dose-response curve was obtained (Figure 4). The possibility that this was due to regional tachyphylaxis to angiotensin was therefore examined in detail since the rises in systemic arterial pressure associated with the intraportal injection of the higher doses of angiotensin, in contrast to the changes in calculated hepatic portal vascular resistance, increased with the increasing intraportal doses of angiotensin.

*Angiotensin tachyphylaxis: repeated injections of angiotensin.* Four successive intraportal injections

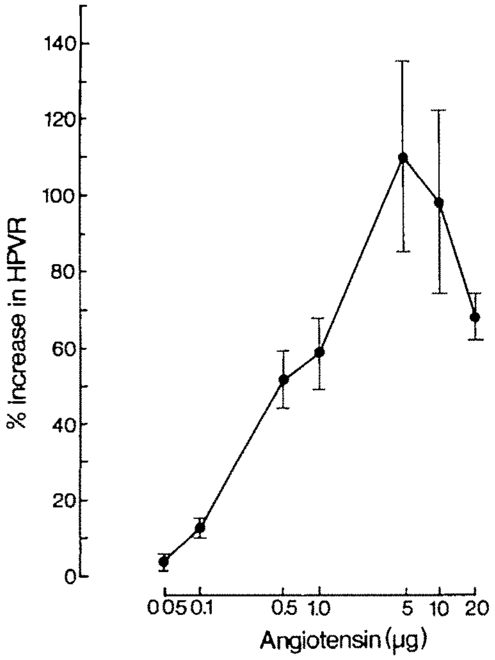

**Figure 4** Dose-response curve for the increase in hepatic portal vascular resistance (HPVR) to intraportal administrations of angiotensin. Abscissa scale: $\log_{10}$ dose of drug in µg; ordinate scale: percentage increase in hepatic portal vascular resistance. Points represent the means of 6 observations, vertical bars show the s.e. means.

of each of five selected doses of angiotensin (0.5, 1, 5, 10 and 20 µg) were made in blocks of equal doses. Within each block, the second, third and fourth injections were made as soon as the hepatic portal perfusion pressure had returned to control levels after the preceding injection. The blocks of injections however were separated by much longer intervals, usually of at least 30 minutes.

This procedure revealed pronounced tachyphylaxis to the repeated intraportal injections of angiotensin. The time course and extent of the development of tachyphylaxis is shown in Figure 5: it is apparent that the tachyphylaxis is most marked between the first and second injections of any of the five selected doses, whilst the increases in HPVR to the fourth injection of each series were very similar to those caused by the third injection.

With each block of injections, test doses of noradrenaline were injected intraportally, to assess whether or not the development of tachyphylaxis was specific to angiotensin: injections of noradrenaline, in contrast to those of angiotensin, showed no sign of the development of tachyphylaxis.

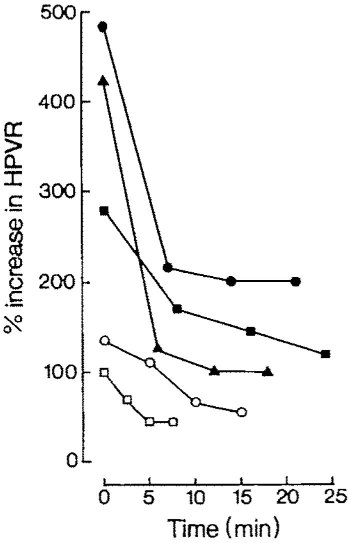

**Figure 5** Diminution in the response of the hepatic portal vascular bed to repeated intraportal injections of angiotensin (0.5 µg □; 1.0 µg ○; 5.0 µg ■; 10.0 µg ▲; 20.0 µg ●). Abscissa scale: time in minutes, showing the intervals between successive injections of equal doses. Ordinate scale: percentage increase in hepatic portal vascular resistance. The injections, four at each dose, were separated by approximately 30 minutes.

That the tachyphylaxis of the hepatic portal vascular bed to angiotensin was a regional variation is illustrated by the fact that the larger intraportal injections of angiotensin, though provoking hepatic portal tachyphylaxis, on reaching the systemic circulation caused mesenteric vasoconstriction judged by the reductions in outflow from the superior mesenteric vein with concomitant increases in systemic arterial pressure. The reductions in mesenteric outflow due to repeated intraportal injections of noradrenaline did not diminish by more than 10% between the first and fourth injections of angiotensin in any series.

Since these experiments revealed clear evidence of a marked portal vascular tachyphylaxis to angiotensin, its influence on the bell-shaped dose-response curve (Figure 4) was ascertained. In one experiment, the interval between successive injections of angiotensin was increased substantially to 5–6 times the duration of the portal vascular response to each injection (i.e. to about 30 min for the larger doses). Although this procedure occupied the entire experiment, the influence of tachyphylaxis was much less apparent, and intraportal injections of 0.1, 0.5, 1, 5, 10 and 20 µg of angiotensin provoked rises in the calculated HPVR of 25, 100, 133, 280, 425 and 485% respectively. Under

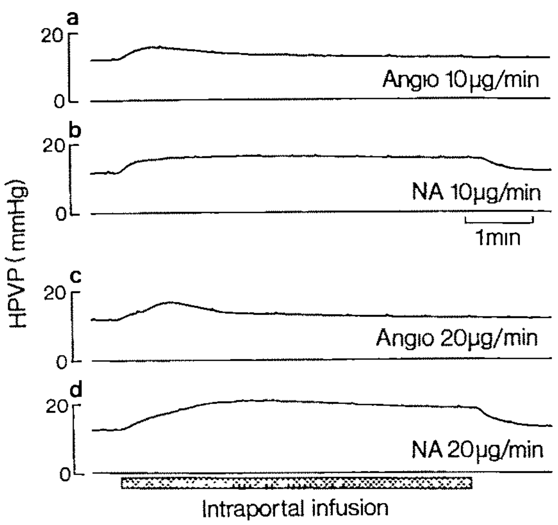

**Figure 6** The effects of intraportal infusions of (a) angiotensin (Angio) 10 µg/min, (b) noradrenaline (NA) 10 µg/min, (c) angiotensin 20 µg/min, and (d) noradrenaline 20 µg/min on the hepatic portal perfusion pressure (HPVP) at constant hepatic portal blood flow. The infusions were maintained for 5 min as shown by the horizontal bar.

these conditions, the dose-response curve for angiotensin on the hepatic portal vascular bed assumed the shape typical of substances such as noradrenaline and adrenaline for which there was, in these experiments, no evidence of tachyphylaxis.

*Angiotensin tachyphylaxis: intraportal infusions of angiotensin.* The specificity of the portal vascular development of tachyphylaxis was further demonstrated by intraportal infusions of both angiotensin and noradrenaline; typical responses are illustrated in Figure 6. The intraportal infusion of 10.0 µg/min of noradrenaline elicited an increase in the hepatic portal perfusion pressure at constant inflow and IVCP, resulting in a peak increase in the calculated HPVR of 33% which was maintained throughout the period of the infusion. Only at higher infusion rates (20 µg/min) was there any sign of 'escape' from the effects of noradrenaline: one such infusion caused a rise in calculated HPVR of 70% which declined to 75% of this value by the end of a 5 min infusion (Figure 6b & d).

In contrast, intraportal infusions of angiotensin (10 µg/min, 20 µg/min) always showed 'escape' from the peak increases in portal vascular resistance: as shown in Figure 6, when 10.0 µg/min was infused, the peak increase of 27% in the calculated HPVR had receded so that the portal resistance returned to control values within 2 min, despite the continued angiotensin infusion (Figure 6a & c). There was no change in the portal vascular resistance when the angiotensin infusion was discontinued at the end of the 5 minutes. Further evidence of the regional variation of the development of tachyphylaxis to angiotensin is the fact that the rises in systemic arterial pressure of 15 and 20 mmHg which accompanied the intraportal infusions of 10 and 20 µg/min respectively of angiotensin were maintained throughout the period of the infusions.

## Discussion

The technique of portal vein perfusion used in the present experiments is similar in principle to that employed by Green, Hall, Sexten & Deal (1959), and Shoemaker (1964); the technique ensures that the portal venous vasculature is perfused with blood derived from its normal supply, the superior mesenteric vein, and permits the quantitative examination of the physiological and pharmacological responses of the hepatic portal venous vasculature uncomplicated by the concomitant responses of the series-coupled vascular circuits of the intestine, pancreas and spleen. Once the portal perfusion was established the portal venous pressures and flows were of the same order as those reported previously. At the end of the experiments the livers were of a good red colour and did not show any signs of 'blueness' or swelling. The perfusion continued for 4 to 5 h without change in perfusion pressure and throughout this time the portal vasculature responded quantitatively to intraportal injection of test doses of noradrenaline. After about 5 h perfusion the portal pressure usually increased indicating an increased basal portal vascular resistance; the experiments were then terminated.

In the present experiments, the intraportal injections of both noradrenaline and adrenaline caused, over a considerable part of the dose-response curve, systemic effects of changes in arterial pressure and heart rate resulting from entry of the catecholamines into the systemic arterial supply. That the effects we describe here are the primary actions of catecholamines on the portal vasculature and not secondary effects arising from alterations in hepatic arterial flow after passage through the cardiopulmonary circuit is indicated by the time course of the peak responses: the peak changes in portal perfusion pressure resulting from intraportal noradrenaline or adrenaline preceded the peak systemic effects by 10 to 15 s, and could not, therefore, arise as secondary effects on the hepatic portal vasculature resulting from systemic effects of the catecholamines after passage through the liver.

Previous reports of the effects of single injections of these catecholamines (Green *et al.*, 1959; Shoemaker, 1964) are thereby confirmed and extended to the establishment of the dose-response relationship. Throughout the dose range investigated, adrenaline was more potent than noradrenaline on both a weight and molar basis, a conclusion reached on isolated preparations of the hepatic portal vein (Hughes & Vane, 1967). Preliminary experiments (Richardson & Withrington, unpublished observations) indicate that paired injections of noradrenaline and adrenaline into the hepatic artery of the dog also cause vasoconstriction, but that in the hepatic arterial vasculature, the relative potency is different from that in the hepatic portal vasculature, noradrenaline being more potent than adrenaline. The effects and the potency order of noradrenaline and adrenaline in the hepatic arterial vascular bed are therefore similar to those in the intestinal (Texter, Chou, Merrill, Laureta & Frolich, 1964; Swan & Reynolds, 1971) and splenic (Davies, Gamble & Withrington, 1973) vascular beds. Systemic release of the catecholamines would therefore cause a profound reduction in total liver blood flow by causing hepatic arterial vasoconstriction (Richardson & Withrington, 1976a) in addition to reducing the inflow to the portal vein by intestinal and splenic vasoconstriction, and causing hepatic portal vasoconstriction.

The present experiments confirm that, in contrast to its action on the hepatic arterial bed, the effects of intraportal injections of vasopressin on the hepatic portal vasculature are weak and somewhat variable, but at high doses dose-dependent reductions in hepatic portal vascular resistance are elicited (Shoemaker, 1964). Isolated preparations of the hepatic portal vein are relaxed by high concentrations (10–100 mu/ml) of vasopressin (Hughes & Vane, 1967), suggesting that the effects seen in the present experiments are due to relaxation of the portal venous vascular smooth muscle.

It is improbable that this effect is of physiological importance, since vasopressin concentrations would be unlikely to approach those shown to be necessary to elicit this effect. Further, it has been known for many years (Clark, 1928) that vasopressin or pituitary extracts cause a reduction in hepatic portal venous pressure which is due to vasoconstriction in the intestinal (Cohen, Sitar, McNeill & Greenway, 1970; McNeill, Stark & Greenway, 1970; Texter *et al.*, 1974) and splenic (Cohen *et al.*, 1970; Davies & Withrington, 1975) vascular beds which occurs at much lower concentrations of vasopressin than are needed to cause reductions in HPVR. The net result of systemic release of vasopressin in the dog is likely to be a reduction in total liver blood flow occasioned by hepatic arterial vasoconstriction (Richardson & Withrington, 1976a) coupled with intestinal and splenic vasoconstriction reducing the hepatic portal inflow and pressure.

Angiotensin injected into the hepatic artery causes a dose-dependent arterial vasoconstriction, responses which remain constant throughout an experiment and are uninfluenced by the interval between successive administrations (Richardson & Withrington, 1976a). Although intraportal injections of angiotensin provoke portal vasoconstriction, in contrast there is a pronounced tachyphylaxis to subsequent intraportal administration. In the present experiments an interval of about 30 min was necessary for the restoration of the portal vascular response to angiotensin subsequent to a single large (10 or 20 μg) intraportal administration. The tachyphylaxis was most apparent over the higher dose range; an observation similar to that of Bock & Gross (1961). The effect of portal tachyphylaxis to angiotensin was even more apparent during intraportal infusions when the portal vascular response declined rapidly after an initial vasoconstriction so that the control portal vascular resistance was restored despite the continued intraportal infusion of the drug. No tachyphylaxis was observed to either intraportal injections or infusions of noradrenaline. Tachyphylaxis to angiotensin has been well established in other vascular territories (Page & Helmer, 1940) and confirmed for angiotensin II amide both *in vitro* (Khairallah, Page, Bumpus & Türker, 1966), and *in vivo* for the pressor response in dogs (Bock & Gross, 1961).

Variations in the development of tachyphylaxis to angiotensin exist between species (Khairallah, *et al.*, 1966) and between different vascular beds in the same species (Jonsson, Svanik & Vikgren, 1967). Jonsson *et al.* (1967) showed that, in the cat, the development of tachyphylaxis to angiotensin was very much more pronounced in the intestinal vasculature than in the hindlimb vessels. In the present experiments, it was consistently observed that intraportal injections of angiotensin which elicited gradually diminishing increases in HPVR were, on reaching the systemic circulation, eliciting almost constant reductions in mesenteric venous outflow and rises in systemic arterial pressure. This implies that the hepatic portal vasculature exhibits this phenomenon to a more marked degree than the intestinal vasculature in the dog.

One consequence of the tachyphylaxis to angiotensin is to ensure an uninterrupted portal blood flow to the liver, which is the main degradative site for angiotensin (Vane, 1969). Whether the portal resistance vessels fail to sustain responses to other vasoactive substances which are destroyed by the liver remains to be established.

The present experiments demonstrate that vasoactive substances either injected systemically or released by physiological mechanisms into the systemic circulation might influence portal vein haemodynamics by a primary action on the portal resistance structures in addition to indirectly affecting the pressure/flow profile of hepatic portal vasculature by direct actions on the series-coupled vascular beds of the intestine, spleen and pancreas. The present experiments reveal, moreover, that both the qualitative and quantitative aspects of the actions of adrenaline, noradrenaline, vasopressin and angiotensin on the portal resistance structure are different from those on the hepatic arterial resistance vessels.

This study was supported by the Medical Research Council and the Smith, Kline & French Foundation; we thank Miss June Pyke and Miss Dorinda Lobendhan for technical assistance.

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*(Received June 16, 1976.*
*Revised September 23, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 303–309

# THE EFFECT OF CONFORMATIONALLY RESTRICTED AMINO ACID ANALOGUES ON THE FROG SPINAL CORD *in vitro*

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**1** The isolated spinal cord of the frog (*Rana pipiens*) was used to examine the structural requirement for the activity of neutral amino acids. The potencies of the aliphatic amino acids, γ-aminobutyric acid (GABA), β-alanine and glycine were compared with the potencies of conformationally restricted cyclopentane and cyclohexane amino acid analogues. Both motoneurone hyperpolarizing and primary afferent depolarizing activity were examined in this study.

**2** On motoneurones β-alanine was the most potent aliphatic amino acid and glycine the least potent. Of the substituted aminocyclopentane carboxylic acids, that compound with a separation of amino and carboxylic acid groups closest to that of the extended GABA molecule (4.74 Å) had a potency similar to GABA. As the separation decreased the hyperpolarizing activity fell off rapidly. The substituted aminocyclohexane carboxylic acids were generally inactive even at a concentration of 10 mM.

**3** Strychnine blocked the motoneurone hyperpolarizing responses to all compounds with a distance between the amino and carboxylic acid groups of 3.66 Å or less, but did not block the response of compounds with a distance of 4.08 Å or greater. Picrotoxin and bicuculline antagonized all the responses to varying degrees and therefore were of little value in characterizing the responses.

**4** On the primary afferents GABA was the most potent aliphatic amino acid and glycine the least potent. The substituted aminocyclohexane carboxylic acids were generally inactive on primary afferents. The response of the substituted aminocyclopentane carboxylic acid whose separation of amino and carboxylic acid groups was closest to that of the extended GABA molecule was most similar to the GABA response. However, (±)-*cis*-3-aminocyclopentane-carboxylic acid (separation = 4.08 Å), which mimicked the action of GABA on motoneurones, closely mimicked the depolarizing action of β-alanine on primary afferents.

**5** The findings suggest that the hyperpolarizing GABA receptor on motoneurones will accept a molecule whose amino and carboxylic acid groups are separated by a distance of 4.08 Å or greater while the glycine receptor will accept a compound with a distance of 3.66 Å or less. The depolarizing GABA receptors on primary afferents appear to be more selective since they are not activated by (±)-*cis*-3-aminocyclopentane carboxylic acid (separation = 4.08 Å), while the motoneurone receptors are.

## Introduction

The use of a number of convulsant agents which have been found to antagonize the inhibitory effects of amino acids on central neurones (Curtis & Johnston, 1974) has led to the conclusion that most neurones possess two types of receptors for the neutral amino acids: one which accepts short chain amino acids, e.g., glycine, and the other which accepts longer chain amino acids, e.g., γ-aminobutyric acid (GABA) (Curtis & Johnston, 1974). Thus strychnine blocks the action of glycine-like amino acids, while bicuculline and picrotoxin block the GABA-like amino acids. Recent studies on the binding of amino acids and convulsants to brain homogenates provide additional support for this hypothesis (Snyder & Bennett, 1976). On primary afferents the depolarizing responses to the neutral amino acids are somewhat more complex than those reported for central neurones in that strychnine primarily antagonizes amino acids in which the negative and positive charged moieties are separated by two carbon atoms, e.g. β-alanine and taurine (Barker, Nicoll & Padjen, 1975a), there being little effect on the GABA and glycine depolarizations.

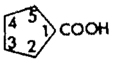
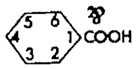
Since the major difference in these amino acids is the number of carbon atoms between the amino and carboxyl group, it is generally agreed that it is the distance between the two charged groups that is of primary importance in conferring the characteristic pharmacological properties on these amino acids. The fact that these amino acids can exist in numerous molecular conformations in aqueous solution have led investigators to use a number of analogues, including conformationally restricted anlogues to determine the conformation of GABA which is physiologically active on a variety of preparations (McGeer, McGeer & McLennan, 1961; Beart, Curtis & Johnston, 1971; Bowery & Brown, 1974; Walker, Azuza, Kerkut & Woodruff, 1975; Segal, Sims & Smissman, 1975; Johnston, Curtis, Beart, Game, McCulloch & Twitchin, 1975; Krogsgaard-Larsen, Johnston, Curtis, Game & McCulloch, 1975; Bowery & Jones, 1976). These investigations have all led to the conclusion that it is the extended form of GABA that is physiologically active. Presumably, as the distance between the amino and carboxyl group is diminished, the amino acid loses its ability to activate GABA receptors and acquires the ability to activate glycine receptors. However, a systematic investigation of this presumption, with a series of conformationally restricted analogues has not been carried out.

The present study compares the effects of a series of aminocyclohexane carboxylic and aminocyclopentane carboxylic acids to the effects of the neutral amino acids, GABA, β-alanine, and glycine in the frog spinal cord. The results obtained on motoneurones have also been compared with the results obtained on pimary afferents to determine if differences exist in the amino acid receptors at these two sites. The use of these analogues on membranes which are considered to have both GABA and glycine receptors should provide insight into the dimensions required for activating these two receptors.

## Methods

Techniques similar to those previously described were used (Barker, *et al.*, 1975a). The drug responses were recorded from the spinal roots of the isolated hemisected frog spinal cord with sucrose gap recordings. The drugs were made up in frog Ringer solution and the pH was adjusted when necessary to 7.3 with HCl or NaOH. All experiments were done at a temperature of 9–13°C which was achieved by passing the Ringer solution and drug solution through a thermoelectric cooling unit before entering the sucrose gap chamber. The use of low temperatures increased the size and stability of the motoneurone hyperpolarizing responses. To determine the relative potency of the various compounds to GABA, a concentration of GABA was selected that caused a 20–40% of maximal response. The concentrations of the other compounds were then varied until a response of equal magnitude was obtained (cf. Figure 1). By using concentrations considerably below those causing a maximal response, it was possible to minimize contamination of the motoneurone hyperpolarizing responses by depolarizing components, which require higher concentrations (cf.

**Table 1** Characteristics of the compound investigated and summary of the experimental data

| | | *Potency relative to GABA* | |
|---|---|---|---|
| *Compounds* | *Distance in Å between amino N and carboxy C atoms*+ | *Monotoneurones (Hyperpolarizing)* | *Primary afferents (Depolarizing)* |
| A. Natural amino acids | $H_2N(CH_2)_nCOOH$ | | |
| Glycine (n = 1) | 2.35 | 0.2 ± 0.08 | 0.07 ± 0.03 |
| β-Alanine (n = 2) | 3.66 | 2.1 ± 0.5 | 0.55 ± 0.21 |
| γ-Aminobutyric acid (n = 3) | 4.74 | 1 | 1 |
| B. Cyclopentanecarboxylic acids |  | | |
| (±)-*cis*-2-amino | 2.54 | < 0.002 | < 0.005 |
| (±)-*trans*-2-amino | 3.50 | 0.02 ± 0.01 | < 0.005 |
| (±)-*cis*-3-amino | 4.08 | 0.37 ± 0.13 | 0.61 ± 0.17 |
| (±)-*trans*-3-amino | 4.77 | 1.4 ± 0.22 | 4.25 ± 1.3 |
| C. Cyclohexanecarboxylic acids |  | | |
| (±)-*cis*-2-amino | 2.81 | < 0.002 | < 0.005 |
| (±)-*trans*-2-amino | 2.70*, 3.47** | < 0.002 | < 0.005 |
| (±)-*cis*-3-amino | 4.81*, 2.46** | 0.002 | 0.02 ± 0.01 |
| (±)-*cis*-4-amino | 4.62 | < 0.002 | < 0.005 |
| (±)-*trans*-4-amino | 5.58*, 4.34** | < 0.002 | < 0.005 |

+ from Segal, *et al.*, 1975, calculated from Dreiding models; * equatorial equatorial; ** axial axial.

Nicoll, Padjen & Barker, 1976). This was particularly true for glycine responses. Magnesium sulphate (10 mM) was added to the Ringer solution to block synaptic transmission in all the experiments except those in which the effect of drugs on root potentials were examined. The following drugs were used in the present study; β-alanine (Aldrich), γ-aminobutyric acid (Aldrich), glycine (Aldrich), glutamate (K & K), bicuculline (K & K). The aminocyclohexane carboxylic acid and aminocyclopentane carboxylic acid compounds were synthesized by Dr L. Maggiora, University of Kansas, Department of Medicinal Chemistry.

## Results

### *Motoneurone response*

The relative hyperpolarizing potency of β-alanine, glycine and the active cyclopentane analogues to GABA were determined in 7 preparations and are summarized in Table 1 and Figure 1. For the aliphatic amino acids β-alanine was the most potent and glycine the least. The (±)-1,3-*trans*-cyclopentane analogue was 1.4 times more potent than GABA and the response had a rapid onset and recovery, similar to that of GABA. The (±)-1,3-*cis*-cyclopentane analogue was 0.37 times as potent as GABA and had a slow onset and recovery. The (±)-1,2-*trans*-cyclopentane analogue was 0.012 times as potent as GABA. The (±)-1,2-*cis*-cyclopentane analogue was inactive at a concentration of 10 mM.

The cyclohexane analogues were all inactive at a concentration of 10 mM, except for the (±)-1,3-*cis*-cyclohexane analogue which was 0.002 times as potent as GABA (Table 1).

### *Primary afferent responses*

Similar responses were obtained on the primary afferents except for a few important differences (Figure 2 and Table 1). On the primary afferents GABA was the most potent aliphatic amino acid and glycine the least potent (see also Barker, *et al.*, 1975a). The (±)-1,3-*trans*-cyclopentane analogue was 4.25 times as potent as GABA and had a similar rapid onset and decline of the response, and also exhibited a fade during the application, which is typical of the GABA response. At the low temperature used in these experiments there was usually a distinct hyperpolarizing component to the β-alanine (and glycine) response. This is best seen in Figure 5. The (±)-1,3-*cis*-cyclopentane analogue was 0.61 times as potent as GABA and reached a maximum considerably more slowly than GABA, but similarly to that seen for the β-alanine response. Furthermore, the depolarizing response was invariably followed by a prolonged hyperpolarization. Except for a small hyperpolarization in 2 preparations at a concentration of 10 mM, the (±)-1,2-*trans*-cyclopentane analogue was inactive, as was the (±)-1,2-*cis*-cyclopentane analogue.

The cyclohexane analogues were all inactive at a concentration of 10 mM, except for (±)-1,3-*cis*-cyclohexane analogue which was 0.02 times as potent as GABA (Table 1) and had no hyperpolarizing component.

### *Effect on root potentials*

The compounds which proved to be inactive on motoneurones and primary afferents were also tested

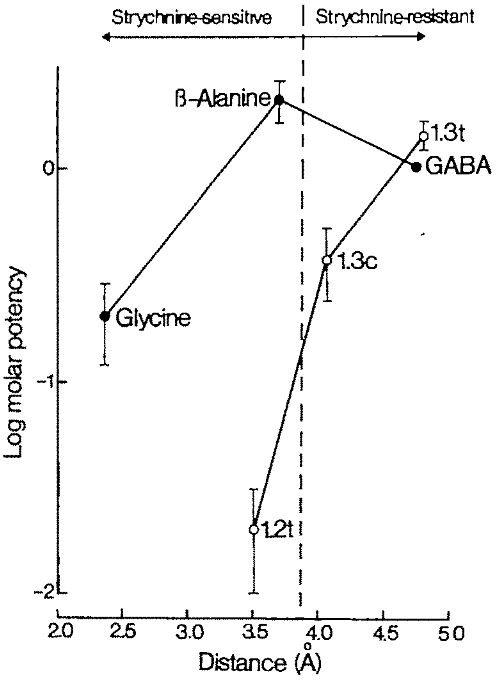


**Figure 1** Comparison of the hyperpolarizing potency of aminocyclopentane carboxylic amino acids with aliphatic amino acids of frog motoneurones. The ordinate scale represents the log molar potency relative to γ-aminobutyric acid (GABA) and the abscissa scale is the distance in Å between the amino and carboxy functions; the value for the aliphatic amino acids is for the fully extended molecule. Compounds to the left of the broken line are blocked by strychnine, while those to the right are not affected by concentrations which entirely block the sensitive compounds. The abbreviations are 1,2t, (±)-1,2-*trans*-aminocyclopentane carboxylic acid; 1,3c, (±)-1,3-*cis*-aminocyclopentane carboxylic acid; 1,3t, (±)-1,3-*trans*-aminocyclopentane carboxylic acid.

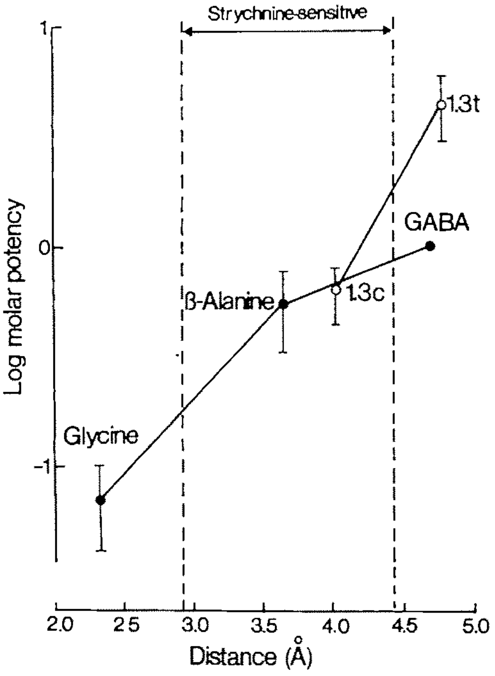


**Figure 2** Comparison of the depolarizing potency of aminocyclopentane carboxylic amino acids with aliphatic amino acids on frog primary afferents. The distinction between strychnine-sensitive and strychnine-resistant responses is not as strong as for motoneurone hyperpolarizing responses, since concentrations of strychnine which completely block β-alanine responses also reduce to some extent the (±)-1,3-*trans*-aminocyclopentane carboxylic acid response. See legend to Figure 1 for further details and abbreviations.

on dorsal root potentials and ventral root potentials. It is possible that these compounds might affect the excitability of interneurones, if the receptors on these cells differed from those examined above. If this were the case then one would expect a depression of the polysynaptic ventral root potential (cf. Curtis, Phillis & Watkins, 1961). In addition, if these compounds were able to block amino acid receptors or block amino acid uptake, one might expect an antagonism of dorsal root potentials in the former case or a prolongation of these potentials in the latter case, since neutral amino acids are thought to mediate dorsal root potentials (Barker & Nicoll, 1972; Davidoff, 1972; Barker *et al.*, 1975a, b). The following cyclohexane analogues were examined at a concentration of 5 mM; (±)-1,2-*cis*, (±)-1,2-*trans*, (±)-1,4-*cis* and (±)-1,4-*trans*. All of these compounds were found to have little effect on either the dorsal or ventral root potentials.

### *Effects of antagonists on motoneurone responses*

Concentrations of strychnine which antagonized the hyperpolarizing responses to glycine and β-alanine also antagonized the hyperpolarizing response of the (±)-1,2-*trans*-cyclopentane analogue (Figure 3a). There was no effect on the (±)-1,3-*trans*-cyclopentane analogue with concentrations of strychnine which completely blocked the above responses. The (±)-1,3-*cis*-cyclopentane analogue response was generally not affected by strychnine, although the response in Figure 3a was slightly reduced by strychnine.

In three preparations with particularly good hyperpolarizations to β-alanine, the action of strychnine on dose-response curves was examined. However, the results were difficult to interpret because of the underlying depolarizing component to the β-alanine response, which is more prominent with high concentrations and is less sensitive to strychnine than the hyperpolarizing component (Nicoll, *et al.*, 1976).

The results obtained with picrotoxin and bicuculline were of little value in characterizing the amino acid responses, because their actions were so variable and, in general, nonspecific (cf. Nicoll, *et al.*, 1976). In Figure 4a picrotoxin antagonized all of the hyperpolarizations with a somewhat greater effect on the β-alanine and the (±)-1,3-*cis*-cyclopentane analogue response. In 2 other preparations the β-alanine responses was slightly less sensitive to picrotoxin than the other 3 responses. Bicuculline proved to be equally nonspecific in its action (Figure 4b).

### *Effects of antagonists on primary afferent responses*

Picrotoxin antagonized the depolarizing responses to β-alanine and the (±)-1,3-*cis*-cyclopentane analogue to the same extent (Figure 5). If there were a hyperpolarizing component to the β-alanine response it was less sensitive to picrotoxin, but could be blocked with high concentrations (0.4 mM). On the other hand, the hyperpolarizing response to the (±)-1,3-*cis*-cyclopentane analogue was quite resistant to the action of picrotoxin. The sensitivity of the (±)-1,3-*trans*-cyclopentane analogue to picrotoxin lay between that of GABA on the one hand and β-alanine and (±)-1,3-*cis*-cyclopentane analogue on the other (Figure 5).

The depolarizing responses to the (±)-1,3-*cis*-cyclopentane analogue had a similar sensitivity to strychnine as did the β-alanine response (Figure 3b). However, the hyperpolarizing component of the β-alanine (and glycine) response was blocked by strychnine, while the hyperpolarizing response to (±)-1,3-*cis*-cyclopentane analogue was entirely resistant. The action of strychnine on the (±)-1,3-*trans*-cyclopentane response was quite variable, but in all 5 preparations, some degree of antagonism was present. However, it was considerably less sensitive to

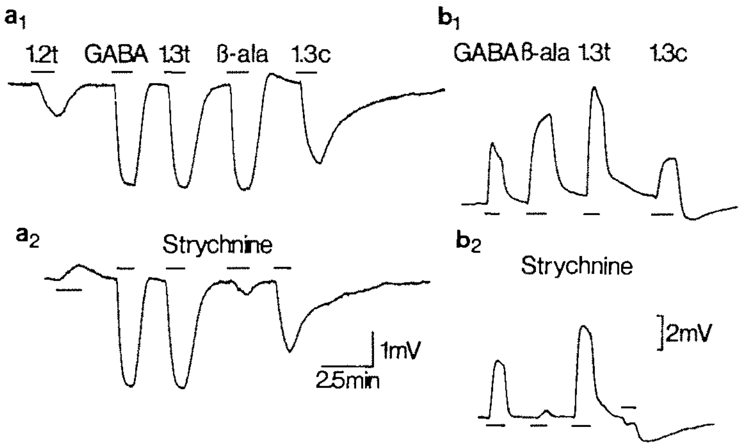


**Figure 3** Effect of strychnine on motoneurone and primary afferent responses: (a1) shows the control ventral root responses to 1,2-*trans*-aminocyclopentane carboxylic acid (1,2t, 5 mM), $\gamma$-aminobutyric acid (GABA, 0.4 mM), 1,3-*trans*-aminocyclopentane carboxylic acid (1,3t, 50 $\mu$M), $\beta$-alanine ($\beta$-ala 0.4 mM), and 1,3-*cis*-aminocyclopentane carboxylic acid (1,3c, 0.5 mM). The record in (a2) was begun 15 min after changing to a Ringer solution containing 1 $\mu$M strychnine. (b1) Shows the control dorsal root responses to GABA (0.5 mM), $\beta$-alanine ($\beta$-ala, 1 mM), 1,3t (0.5 mM) and 1,3c (1 mM). The record in (b2) was begun 20 min after starting strychnine (20 $\mu$M). The time calibration in (a) also applies to (b).

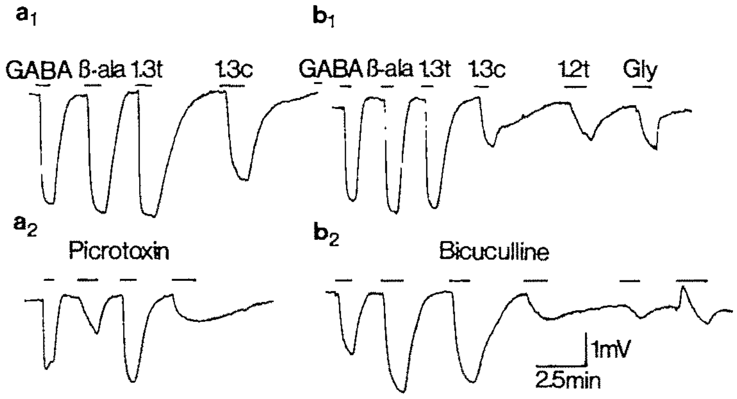


**Figure 4** Effect of picrotoxin and bicuculline on motoneurone responses: (a1) shows the control responses to $\gamma$-aminobutyric acid (GABA, 0.2 mM), $\beta$-alanine ($\beta$-ala, 0.1 mM), 1,3-*trans*-aminocyclopentane carboxylic acid (1,3t, 0.4 mM) and 1,3-*cis*-aminocyclopentane carboxylic acid (1,3c, 1 mM). The responses in (a2) were obtained 10 min after starting a Ringer solution containing 5 $\mu$M picrotoxin. The control responses in (b1) were obtained with the following concentrations GABA (0.2 mM), $\beta$-alanine ($\beta$-ala, 0.2 mM), 1,3t (0.2 mM), 1,2t (5 mM) and glycine (Gly, 0.2 mM). The responses in (b2) were obtained 15 min after changing to a Ringer solution containing 50 $\mu$M bicuculline. The calibration in (b) also applies to (a).

strychnine than were the $\beta$-alanine and the (±)-1,3-*cis*-cyclopentane analogue responses. At the low temperatures used in these experiments, concentrations of strychnine which completely blocked the $\beta$-alanine response often resulted in a small decrease in the GABA response (10–15%) which did not appear to be due to a gradual deterioration of the preparation.

## Discussion

In the present study an attempt was made to determine the conformational requirements of a compound for activating glycine and GABA receptors in the frog spinal cord. The advantages derived from using aminocyclohexane carboxylic acids and especially aminocyclopentane carboxylic acids in such

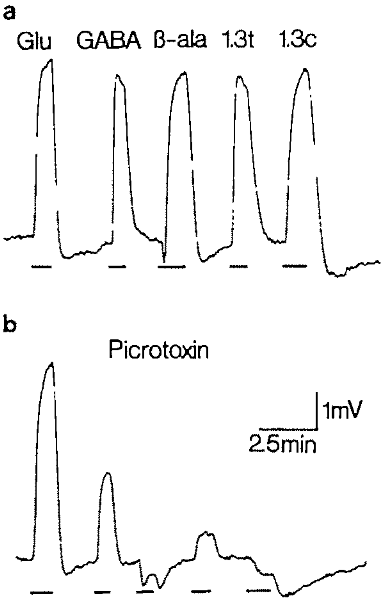


**Figure 5** Effect of picrotoxin on primary afferent responses. The control responses in (a) were obtained with the following concentrations: glutamate (Glu, 1 mM), γ-aminobutyric acid (GABA, 0.4 mM), β-alanine (β-ala, 1 mM), 1,3-*trans*-aminocyclopentane carboxylic acid (1,3t, 80 µM), and 1,3-*cis*-amino-cyclopentane-carboxylic acid (1,3c, 1 mM). The responses in (b) were obtained 15 min after starting a Ringer solution containing 0.1 mM picrotoxin.

a study have been discussed (Segal *et al.*, 1975). It should be kept in mind that the dual action of many of the compounds may have introduced small errors in relative potency, but this problem was minimized by appropriate adjustment of amino acid concentrations. It is also possible that endogenous amino acids may be released from storage sites by structural analogues, as has been shown in the sympathetic ganglion (Bowery, Brown, Collins, Galvan, Marsh & Yamini, 1976). The results with the cyclopentane analogue series on motoneurones proved to be the most informative of this study and indicate that those molecules which have a spatial geometric separation of amino and carboxy groups closest to that of the fully extended GABA molecule (separation=4.74 Å) are most effective in activating GABA receptors. The present findings are qualitatively similar to those of Segal *et al.* (1975) on hippocampal neurones and to other studies with other analogues on a variety of preparations (McGeer, *et al.*, 1961; Beart, *et al.*, 1971; Bowery & Brown, 1974; Walker, *et al.*, 1975; Krogsgaard-Larsen, Johnston, Curtis, Game & McCulloch, 1975; Johnston, *et al.*, 1975; Bowery & Jones, 1976). However, as the distance between the amino and carboxy groups was decreased for the cyclopentane analogues, the potency fell off much more rapidly than in hippocampal neurones, which is surprising in that frog motoneurones are more responsive to shorter chain amino acids than are hippocampal neurones. This might be explained by steric effects that can be expected with substitution of ring structures between the amino and carboxy group which would alter receptor preference independent of the distance between the amino and carboxy group.

Of the amino acid antagonists used in this study, strychnine was the most selective in its action on motoneurones and provided a means for determining whether the analogues activated GABA or glycine receptors. (Although β-alanine was the most potent of the strychnine-sensitive amino acids, the receptor involved in these responses will be referred to as the glycine receptor, in keeping with terminology in the mammalian CNS (Curtis & Johnston, 1974)). All of the active compounds with a distance between the amino and carboxy group of 3.66 Å (as in β-alanine) or less were blocked by strychnine, while those with a distance of 4.08 Å (1,3-*cis*-cyclopentane analogue) or greater were unaffected by concentrations of strychnine which entirely blocked the sensitive responses. These results would imply that on frog motoneurones a very sharp cut-off point exists for compounds which activate GABA receptors and for those that activate glycine receptors. It was hoped that picrotoxin and bicuculline would provide complementary evidence for the findings obtained with strychnine, since Segal *et al.* (1975) reported that these convulsants antagonize the action of the 1,3-*cis*-cyclopentane analogue. However, picrotoxin and bicuculline proved to be of little use in distinguishing between the two receptors, since all of the responses were affected to varying degrees. This nonselectivity on frog motoneurones (cf. Nicoll *et al.*, 1976) is in contrast to results obtained with neurones in the cat CNS (Cf. Curtis & Johnston, 1974).

The results obtained from primary afferents differed considerably from those on motoneurones and are somewhat more difficult to characterize. As seen with motoneurones the cyclopentane analogue (i.e., 1,3-*trans*), with dimensions closest to that of the fully extended GABA molecule, most closely mimicked the response to GABA except that strychnine antagonized the response to some extent. Unlike the findings on motoneurones the depolarizing response to the (±)-1,3-*cis*-cyclopentane analogue had properties similar to the β-alanine response rather than the GABA response. The time course, potency and sensitivity to strychnine and picrotoxin of the depolarizations to these two compounds were remarkably similar. However, the hyperpolarizing component to the β-alanine (and glycine) response was blocked by strychnine whereas that elicited by the 1,3-*cis* compound was not. In addition, as reported earlier (Barker *et al.*, 1975a), the depolarizing response to glycine was not blocked by strychnine.

It is not clear why most of the cyclohexane analogues which have distances between their amino and carboxy groups comparable to those for the aliphatic amino acids in their fully extended form and to the cyclopentane analogues showed little activity. Interestingly, Segal *et al.* (1975) found the cyclohexane analogues to be weakly active on hippocampal neurones. Furthermore, these compounds had little effect on root potentials which are thought to involve the release of neutral amino acids (Barker & Nicoll, 1972; Davidoff, 1972; Barker *et al.*, 1975a, b) suggesting that they neither compete for neutral amino acid receptors nor uptake sites.

In summary, the major difference between the hyperpolarizing motoneurone responses and the primary afferent depolarizing responses is that the strychnine sensitive receptors on motoneurones will accept a compound with a distance between the amino and carboxy functions of 3.66 Å or less, whereas the strychnine senstive depolarizing receptors on primary afferents will accept a compound with a distance larger than 2.35 Å (glycine) and smaller than 4.74 Å (GABA). However, the selectivity of strychnine is not as great as on primary afferents since the response to the (±)-1,3-*trans*-cyclopentane analogue (separation = 4.77 Å) is affected to some extent.

I wish to thank Dr L. Maggiora for synthesizing the compounds used in the present study and Dr M. Segal for his gift of (±)-1,3-*trans*-cyclopentane and (±)-1,2-*cis*-cyclopentane. The critical reading of the manuscript by Dr A. Trevor is appreciated. This work was supported by a PMAF Starter Grant and a grant from the Academic Senate of the University of California, San Francisco.

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*(Received June 17, 1976.*
*Revised September 26, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 311–322

# THE HAEMODYNAMIC AND METABOLIC EFFECTS OF MG 8926, A PROSPECTIVE ANTIDYSRHYTHMIC AND ANTIANGINAL AGENT

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1 The antidysrhythmic, haemodynamic and metabolic effects of a new prospective antianginal and antidysrhythmic agent, N-(3,3-diphenylpropyl)-$\alpha$-methyl-$\beta$-cyclohexylethylamine hydrochloride (MG 8926), have been compared with the chemically related substance, prenylamine, in anaesthetized greyhounds and guinea-pigs.

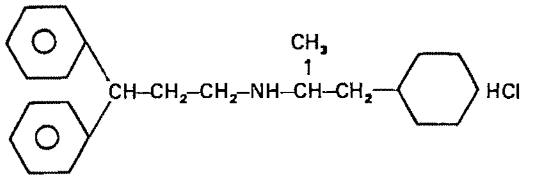
2 When given intravenously 20 min beforehand, both MG 8926 and prenylamine (5 mg/kg) significantly suppressed the early dysrhythmias induced by coronary artery ligation in anaesthetized greyhounds. At a dose of 1 mg/kg, MG 8926 also protected anaesthetized guinea-pigs from dysrhythmias induced by ouabain infusions.

3 In dogs pretreated with MG 8926, metabolic changes indicative of myocardial ischaemia (increased $P\text{CO}_2$ and potassium efflux, decreased oxygen content and pH) were less marked than those occurring in control animals.

4 Evidence was obtained that MG 8926, when given either before or after coronary occlusion, was capable of decreasing the severity of myocardial ischaemia as assessed by ST-segment changes in epicardial electrocardiograms.

## Introduction

MG 8926 is a recently synthesized secondary amine structurally related to the antianginal drug prenylamine. It increases coronary flow in the isolated guinea-pig heart and partially inhibits pitressin-induced ST-segment changes in anaesthetized rats (M. Carissimi, personal communication 1975). In preliminary experiments we found that it also elevated coronary blood flow in the anaesthetized greyhound and reduced the incidence of cardiac dysrhythmias which result from acute ligation of the anterior descending branch of the left coronary artery in this experimental model. This paper describes the detailed haemodynamic effects of this compound and, where appropriate, these effects are compared with those of prenylamine.



N-(3,3-diphenylpropyl)-$\alpha$-methyl-$\beta$-cyclohexylethylamine hydrochloride (MG 8926).

## Methods

### *Anaesthetized greyhounds*

Experiments were carried out on 27 greyhounds of either sex weighing between 23 and 31 kg. Anaesthesia was induced by intravenous administration of sodium thiopentone (20 mg/kg). After endotracheal intubation, respiration was applied from a positive-pressure ventilation pump (25 strokes/min), with 100% $O_2$ containing 0.5–1.0% trichlorethylene. The tidal volume of the pump was adjusted to maintain an arterial $P\text{CO}_2$ of 35–40 mmHg (1 mmHg = 1.33 mbar). Reflex movements were prevented by the intermittent intramuscular administration of suxamethonium chloride (100 mg).

In six of the dogs, myocardial nutritive blood flow was measured without thoracotomy by the radioactive xenon clearance method (Ledingham, McBride, Parratt & Vance, 1970; Marshall & Parratt, 1976). In order to measure myocardial $O_2$ availability and consumption, a catheter was positioned under fluoroscopic control in the coronary sinus and blood samples were taken, without exposure to air, from this catheter and, simultaneously, from a catheter in the descending aorta. The blood was analysed for $P\text{O}_2$, $P\text{CO}_2$ and pH as previously described (Marshall, Parratt & Ledingham, 1974).

In another group of 12 dogs, catheters were placed, under fluoroscopic control, in the descending aorta (via the right femoral artery), the right atrium (via the right saphenous vein), the pulmonary artery and the coronary sinus (both via the left external jugular vein). A catheter-tip transducer (Millar Instruments, Inc., Houston, Texas) was inserted into the lumen of the left ventricle (via the left carotid artery in the neck) for the measurement of left ventricular pressure and also $dP/dt$ (using an Elema-Schönander differentiating circuit). The frequency response of this transducer system is flat to 200 Hz. Records of left ventricular pressure at high gain allowed accurate assessments to be made of left ventricular end-diastolic pressure (LVEDP).

Cardiac output was measured either by dye-dilution, indocyanine green (2.5 mg) being injected as a bolus into the right atrium and blood withdrawn at a constant rate through a Waters densitometer, or by thermo-dilution, as described by Douglas, McDonald, Milligan, Mellon & Ledingham (1975). All cardiac output determinations were made in duplicate. There is a good correlation between cardiac output measurements made by these two methods (Douglas *et al.*, 1975).

In the remaining dogs, the heart was exposed through a left thoracotomy and the pericardium overlying the anterolateral aspect of the heart was incised. Blood flow in the circumflex branch of the left coronary artery was measured with a Nycotron 372 electromagnetic flow meter using a calibrated probe of 1.5–2.5 mm diameter. The anterior descending branch of the left coronary artery (LAD), at a point distal to the septal artery branch, was prepared for ligation with minimum dissection. A major branch of the main vein adjacent to the artery (the anterior coronary vein) was catheterized using the Seldinger technique with a 10 cm Longdwel teflon catheter (size 20G). This local coronary vein catheter was not tied in position and was manipulated until its tip lay well into the region of the myocardium which was to be made ischaemic. It has been shown that such a coronary vein catheter, after coronary artery ligation, drains blood predominantly from the ischaemic region (Fisher, Heimbach, Ledingham, Marshall & Parratt, 1973; Marshall *et al.*, 1974).

Blood samples were taken, without exposure to air, at regular intervals and were analysed for $O_2$ and $CO_2$ tensions, $O_2$ content and pH, as outlined by Ledingham *et al.* (1970), except that the value used for haemoglobin oxygen-binding capacity was 1.39. Blood samples from the aorta, coronary sinus and coronary vein were taken immediately before and 30 min after coronary artery ligation and were analysed for lactate (Hohorst enzymatic method using a Boehringer test combination) and for plasma potassium by standard flame photometry.

After a 20–25 min stabilization period, the ligature on the coronary artery was tied in one stage and the number of ventricular ectopic beats counted during each 5 min period for a total period of 30 min; no blood samples were taken during this time since manipulation of the coronary venous catheter sometimes itself induced arrhythmias. No arrhythmias occurred in any of the animals after 30 minutes. The arrhythmia counts obtained in these groups of dogs were compared with those previously obtained in untreated animals (Marshall & Parratt, 1974).

After the crucial 30 min period immediately after ligation, seven silver epicardial electrodes embedded in a band of rubber were sutured to the surface of the anterior left ventricular wall, so that at least four electrodes were situated in the obviously ischaemic zone. Frequent epicardial ECG recordings were taken before and at various times after drug infusion. A Portex nylon catheter (o.d. 1.34 mm) was then inserted into the peripheral stump of the ligated artery and was used for the measurement of peripheral coronary pressure (that is back pressure from the ischaemic zone measured with a capacitance transducer) and peripheral coronary blood flow (retrograde flow from this region).

At the end of each experiment, a bolus of diffusible dye was injected at a pressure of 50 mmHg into the peripheral stump of the ligated coronary artery. Fibrillation was immediately induced with potassium chloride and the dyed muscle quickly excised and weighed. The mass of this dyed ischaemic area of muscle was expressed as a percentage of the free ventricular wall.

Systemic arterial pressure (pulsatile and mean by electronic integration), mean right atrial pressure, pulmonary artery pressure, left ventricular pressure and $dP/dt$, LVEDP, left circumflex coronary blood flow and the electrocardiogram (standard limb lead II) were recorded on an Elema-Schönander ink-jet writing recorder (Mingograph 81). Myocardial $O_2$ extraction was calculated as outlined by Marshall & Parratt (1973) and cardiac work, peripheral vascular resistance, and whole body $O_2$ consumption as described by Ledingham, Parratt, Smith & Vance (1971).

In four additional dogs, the effects of prophylactically administered MG 8926 on ischaemia-induced epicardial ST-segment changes were investigated. These dogs were subjected to thoracotomy and a nylon snare placed around the LAD. Nine silver electrodes embedded in a triangular sheet of rubber were sutured on to the anterior surface of the left ventricle; epicardial electrocardiograms could be recorded simultaneously from any three sites through a rapid switching circuit. After control tracings from each site had been obtained the LAD was occluded and epicardial ECGs recorded from each site after 1, 2 and 3 min of occlusion. The snare was then released and the artery reoccluded after a rest period of at least

20 minutes. After two or three control occlusions the procedure was repeated 10 and 30 min after the administration of MG 8926 (5 mg/kg). Short 3 min occlusions were used in this series of animals, since releasing the occlusion after more than 3 min caused marked ventricular tachycardia and ventricular fibrillation.

All results were statistically analysed using Student's *t* test for paired or unpaired data. MG 8926 (as the hydrochloride) and prenylamine (as the lactate) were dissolved in warm distilled water and infused slowly into a femoral vein. All doses in the text refer to the salts.

### *Anaesthetized guinea-pigs*

The preparation used was essentially that described by Dohadwalla, Freedberg & Vaughan Williams (1969). Female guinea-pigs weighing between 300–400 g were anaesthetized with urethane (1.6 g/kg, i.p.). Artificial respiration was applied using a Palmer small-animal pump with a stroke volume of 2–3 ml; the rate was 25 per minute. Rectal temperature was monitored with an Ellab thermocouple and the electrocardiogram (limb lead I or II) continuously monitored on an oscilloscope. Ouabain was infused into a jugular vein at a rate of 8 µg/min for 30 s every 2 minutes. At the end of every 2 min period the electrocardiogram was recorded for 10 s on a Mingograph 81 ink-jet writing recorder. The amounts of ouabain required to produce (a) unequal R-R intervals, (b) ventricular extrasystoles, (c) sustained ventricular tachycardia, (d) ventricular fibrillation and (e) cardiac arrest were noted. Five minutes before starting the ouabain infusion, animals were given intravenous 0.9% w/v NaCl solution (saline), MG 8926 or prenylamine. All injection volumes were less than 1 ml and control (saline) experiments were conducted every day to minimize any seasonal variations in the animals' responses.

### *Isolated electrically-driven atrial muscle of guinea-pig*

Guinea-pigs weighing between 200–310 g were killed by stunning and exsanguination and the left atrium quickly excised and placed in a 10 ml organ bath containing Krebs–Henseleit solution (g/1: NaCl 6.9, KCl 0.35, $NaHCO_3$ 2.1, $KH_2PO_4$ 0.16, $MgSO_4$ 0.29, $CaCl_2$ 0.56, glucose 2.0) bubbled with carbogen. The left atrial preparations were stimulated via a platinum electrode with supra-threshold (3–6 V) square wave pulses, of 5 ms duration and at 2 Hz. Contractions were measured by attaching the free end of the atria by means of a cotton thread to a strain gauge transducer (Ether UF1) and were recorded on a Devices M2 recorder.

To measure the effective refractory period of left atria, the stimulation was steadily increased until the atria could no longer follow each stimulus. This maximum driving frequency (MDF) is inversely related to effective refractory period (Ellis, 1956). In this investigation, control runs (usually two) were carried out at 30 min intervals until consistent MDFs were obtained. The test drug was then added to the organ bath and the procedure repeated either 10 or 20 min later (see Results section). Changes in maximal driving frequency (and thus reciprocal changes in effective refractory period) were expressed as percentages of the control value.

## Results

### *Haemodynamic effects of intravenously administered MG 8926 in the closed chest greyhound*

In order to ascertain the active dose-range of MG 8926, the haemodynamic effects of intravenous doses of 0.5, 2.5 and 5.0 mg/kg were investigated. The results are summarized in Table 1 and show that MG 8926 first caused discernible haemodynamic changes in intravenous doses of about 2.5 mg/kg. In this dose-range, MG 8926 reduced systemic blood pressure within 2–5 s of injection by 15–60 mmHg without significantly changing heart rate. External cardiac work and cardiac output were significantly elevated and, since heart rate remained constant, the increase in cardiac output could be attributed to an increase in stroke volume. Myocardial nutritional blood flow (as assessed by xenon clearance) was consistently elevated by MG 8926 (2.5 and 5.0 mg/kg) and, since systemic blood pressure was reduced, this indicates a reduction in coronary vascular resistance of between 30–55%. Since coronary sinus $P_{O_2}$ rose (and therefore myocardial $O_2$ extraction fell) there was no change in myocardial $O_2$ consumption, and thus the ratio of available $O_2$ to consumed $O_2$ was increased by MG 8926 (from $2.7 \pm 0.6$ to $4.0 \pm 0.6$; $P < 0.05$). All these haemodynamic effects of MG 8926 were relatively transient and all parameters returned to control values within 10–15 min of injection.

### *A comparison of the haemodynamic effects of intravenously administered MG 8926 and prenylamine lactate (5 mg/kg) in the open chest anaesthetized greyhound*

After the intravenous injection of MG 8926 (5 mg/kg; 6 dogs) there occurred an immediate transient decrease in blood pressure (mean decrease in diastolic pressure of $44 \pm 7$ mmHg). Neither heart rate (mean change + $8 \pm 14$ beats/min) nor left ventricular d*P*/d*t max* (mean change + $80 \pm 140$ mmHg/s) was affected by the drug. In the two experiments in which they were measured, cardiac output and myocardial blood flow were considerably but transiently increased (by

21% and by 68% respectively) and there was a marked reduction in myocardial $O_2$ extraction (from 41 ± 6 to 21 ± 5%; $P<0.05$) 3 min after injection. The haemodynamic effects of MG 8926 in the open chest animal were, like those in the closed chest, transient so that 10 min after drug administration there were no significant changes in blood pressure (185 ± 12 mmHg systolic and 128 ± 6 mmHg diastolic before the drug and 187 ± 12 and 133 ± 6 mmHg after), heart rate (187 ± 12 to 169 ± 7), left ventricular $dP/dt$ *max* (3750 ± 420 to 3580 ± 40 mmHg/s), LVEDP (8 ± 1 to 9 ± 1 mmHg), cardiac output (4.1 ± 0.4 to 4.0 ± 0.4 l/min), external cardiac work (4.5 ± 0.5 to 4.9 ± 0.6 kgm/min), myocardial $O_2$ extraction (41 ± 6 to 37 ± 6%), peripheral vascular resistance (37 ± 2 to 38 ± 2 arbitrary units) and whole body $O_2$ consumption (134 ± 15 to 114 ± 10 ml/minute).

Prenylamine lactate (5 mg/kg; 6 dogs) also caused an immediate fall in blood pressure (a reduction of 46 ± 10 mmHg in diastolic pressure) but in contrast to MG 8926, this was accompanied by transient but significant increases in heart rate (from 168 ± 11 to 202 ± 7 beats/min; $P<0.05$) and in left ventricular $dP/dt$ *max* (mean increase 667 ± 80 mmHg/second). Pulmonary artery pressure was initially increased from 18 ± 3 mmHg systolic, (9 ± 1 diastolic) to 24 ± 4 mmHg systolic (13 ± 1 mmHg diastolic) ($P<0.05$). Like MG 8926, prenylamine lactate also caused a transient increase in cardiac output (4.3 ± 0.2 to 5.5 ± 0.2 litres/min, $P<0.05$) and a marked decrease in myocardial $O_2$ extraction (38 ± 5 to 22 ± 3%; $P<0.05$). As with MG 8926, all parameters had returned to control levels 10 min after the injection of prenylamine.

A constant finding with prenylamine was the production of dysrhythmias on injection. These dysrhythmias were ventricular in origin and lasted 5–10 minutes. In contrast, MG 8926 altered cardiac rhythm, and that transiently, on only one occasion.

### *The effects of MG 8926 and prenylamine lactate on the immediate dysrhythmias ensuing after acute coronary artery ligation*

The results of this investigation are presented and compared with previous results in control animals in Table 2. Ventricular dysrhythmias are common in this preparation especially in the 10–20 min post-ligation period and in 4 out of the 12 control dogs ventricular fibrillation occurred during this time. In contrast, significantly fewer dysrhythmias were seen during this crucial period in dogs pretreated with either MG 8926 or prenylamine, both given in an intravenous dose of 5.0 mg/kg, 20 min before ligation. None of the animals given MG 8926, and only one animal given prenylamine, succumbed to ventricular fibrillation but in 5 of the treated dogs (3 given MG 8926, 2 given prenylamine) there were sustained runs of ventricular

**Table 1** The immediate haemodynamic changes (mean ± s.e.) resulting from the intravenous administration of MG 8926 in anaesthetized closed-chest greyhounds

| | *Dose of MG 8926 (mg/kg)* | | | | | |
|---|---|---|---|---|---|---|
| | *(n=3)*<br>*0.5* | | *(n=6)*<br>*2.5* | | *(n=5)*<br>*5.0* | |
| | *Pre* | *Post* | *Pre* | *Post* | *Pre* | *Post* |
| Systolic blood pressure (mmHg) | 208 ± 8 | 198 ± 16 | 201 ± 8 | 165 ± 12* | 190 ± 8 | 130 ± 10* |
| Diastolic blood pressure (mmHg) | 142 ± 2 | 143 ± 9 | 145 ± 6 | 112 ± 9* | 138 ± 5 | 79 ± 7* |
| Heart rate (beats/min) | 197 ± 27 | 200 ± 31 | 199 ± 10 | 215 ± 8 | 185 ± 11 | 200 ± 13 |
| Cardiac output (1/min) | 2.7 ± 1.4 | 2.7 ± 1.7 | 2.6 ± 0.6 | 3.5 ± 0.6* | 2.6 ± 0.4 | 3.6 ± 0.5* |
| External cardiac work (kgm/min) | 3.0 ± 1.2 | 2.9 ± 1.5 | 3.0 ± 0.7 | 3.5 ± 0.8 | 3.1 ± 0.5 | 4.4 ± 0.4* |
| Peripheral resistance (units) | 78 ± 30 | 93 ± 38 | 73 ± 16 | 50 ± 15 | 68 ± 9 | 38 ± 6* |
| Myocardial blood flow (ml 100 $g^{-1}$ $min^{-1}$) | 112 ± 24 | 101 ± 21 | 99 ± 12 | 121 ± 9* | 110 ± 9 | 133 ± 19* |
| Myocardial oxygen extraction (%) | 44 ± 4 | 46 ± 4 | 46 ± 6 | 38 ± 6 | 53 ± 9 | 34 ± 8* |
| Myocardial oxygen consumption (ml 100 $g^{-1}$ $min^{-1}$) | 14.5 ± 3.1 | 12.7 ± 2.5 | 11.8 ± 2.5 | 12.1 ± 3.4 | 12.5 ± 5.1 | 10.1 ± 6.4 |
| Myocardial vascular resistance (units) | 1.4 ± 0.2 | 1.8 ± 0.3 | 2.0 ± 0.1 | 1.3 ± 0.1* | 1.5 ± 0.1 | 0.9 ± 0.1* |

*$P<0.05$

**Table 2** Analysis of the number of ventricular ectopic beats at various times after acute coronary ligation in control (untreated) dogs and in dogs pretreated with either MG 8926 or prenylamine

| *Group* | *0–5 min* | *6–10 min* | *11–15 min* | *16–20 min* | *21–25 min* | *26–30 min* | *Total* | *Fibrillation* |
|---|---|---|---|---|---|---|---|---|
| Control | 120±42 | 150±60 | 94±67 | 286±92 | 96±38 | 52±21 | 904±260 | 4/12 |
| MG 8926<br>5 mg/kg | 69±34 | 55±29* | 84±59 | 118±82* | 2± 2* | 2± 1* | 329±150* | 0/6 |
| Prenylamine<br>5 mg/kg | 131±32 | 52±47* | 42±31 | 43±23* | 85±54 | 20±19 | 372±196* | 1/6 |

Values are means ± s.e. All dogs breathed 100% $O_2$.
* Significantly different from control; $P < 0.05$.

**Table 3** The haemodynamic effects of acute coronary artery ligation in control dogs and in dogs pretreated with either MG 8926 or prenylamine

| | *Controls* (n=8) | | *MG 8926* (n=6) | | *Prenylamine* (n=6) | |
|---|---|---|---|---|---|---|
| | *Pre* | *30 min post* | *Pre* | *30 min post* | *Pre* | *30 min post* |
| Heart rate (beats/min) | 176± 5 | 182± 6 | 174± 3 | 183± 9 | 160± 10 | 176± 7 |
| Mean blood pressure (mmHg) | 130± 4 | 124± 6 | 152± 9 | 136± 10 | 150± 13 | 137± 13 |
| LV d*P*/d*t max* (mmHg/s) | 2910±280 | 2600±190 | 3350±340 | 2716±490 | 2240±192 | 2100±201 |
| LVEDP (mmHg) | 6± 1 | 11± 2* | 8± 1 | 10± 2* | 7± 2 | 14± 2* |
| Cardiac output (l/min) | 3.2±0.4 | 2.2±0.2* | 3.7±0.3 | 2.7±0.2* | 4.1±0.1 | 3.2±0.3* |
| External cardiac work (kgm/min) | 5.8±0.8 | 3.4±0.5* | 4.7±0.5 | 3.2±0.1* | 5.2±0.3 | 3.3±0.4* |

Values are mean ± s.e.
*$P < 0.05$.

**Table 4** Changes in coronary sinus and coronary vein, $P_{CO_2}$ and pH, and in myocardial $O_2$ extraction, before and 30 min after, coronary artery ligation in control dogs and in dogs treated with either MG 8926 or prenylamine

| | *Controls* | | *MG 8926* | | *Prenylamine* | |
|---|---|---|---|---|---|---|
| | *Pre* | *Post* | *Pre* | *Post* | *Pre* | *Post* |
| *Coronary sinus* | | | | | | |
| $O_2$ extraction (%) | 52±3 | 54±4 | 37±6 | 45±7 | 41±7 | 48±9 |
| $P_{CO_2}$ (mmHg) | 46±2 | 48±2 | 51±4 | 50±4 | 50±2 | 51±2 |
| pH (units) | 7.350±0.016 | 7.345±0.020 | 7.321±0.010 | 7.326±0.013 | 7.344±0.032 | 7.341±0.033 |
| *Coronary vein* | | | | | | |
| $O_2$ extraction (%) | 54±3 | 69±3* | 39±5 | 48±8* | 47±8 | 61±12* |
| $P_{CO_2}$ (mmHg) | 45±2 | 60±3* | 49±4 | 54±3* | 47±8 | 61±5* |
| pH (units) | 7.347±0.014 | 7.265±0.023* | 7.325±0.017 | 7.313±0.009 | 7.350±0.034 | 7.310±0.043* |

Values are mean±s.e.; $n=6$.
*$P<0.05$.

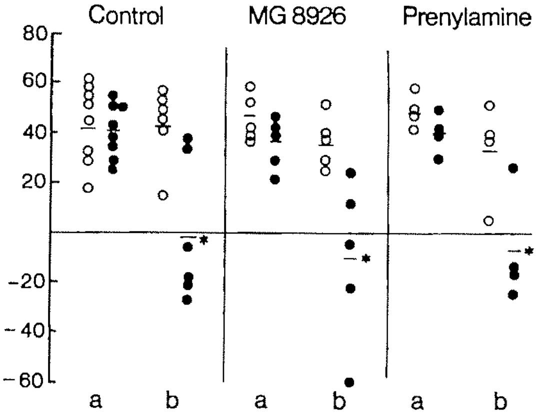


**Figure 1** The effects of coronary artery ligation on lactate extraction coefficients (A–V%) calculated using samples of blood from the coronary sinus (draining both areas of heart; (○) and coronary vein (draining the ischaemic zone; (●), in control dogs and in dogs pretreated with either MG 8926 or prenylamine (5.0 mg/kg). Negative values for A–V indicate lactate production. Samples were taken immediately before (a) and 30 min after coronary artery ligation (b). Note that lactate production was evident in blood draining from the ischaemic myocardium in all three groups of animals.

tachycardia. There were no dysrhythmias in any dog after 30 minutes. The reduced incidence of dysrhythmias in dogs pretreated with MG 8926 or prenylamine was remarkable since the area of the left ventricular wall served by the ligated artery was significantly greater (33 ± 2 and 32 ± 2% respectively) than that in control dogs (21 ± 2%).

### *Haemodynamic and metabolic consequences of acute coronary artery ligation in dogs pretreated with MG 8926 or prenylamine*

The haemodynamic consequences of acute coronary artery ligation in this experimental model have been described previously (Marshall *et al.*, 1974). The haemodynamic effects of ligation in this series of animals, given MG 8926 or prenylamine, were essentially similar (Table 3) and included a decrease in cardiac output and external cardiac work. These changes are probably secondary to a decreased cardiac contractility, as manifested by transient decreases in left ventricular $dP/dt$ *max* with more sustained increases in LVEDP (Table 3).

When a major branch of the left anterior descending coronary artery is ligated, metabolic changes occur in the venous blood draining the acutely ischaemic region which do not occur in blood draining the remaining, essentially normal areas; these changes in untreated dogs have been described in detail by Ledingham *et al.* (1974) and include reductions in coronary venous $Po_2$, $O_2$ content, pH and lactate extraction and an increase in $Pco_2$. Changes in these parameters induced by coronary artery ligation in dogs pretreated with either MG 8926 or prenylamine (5.0 mg/kg) are shown in Table 4, and the effects on lactate extraction illustrated in Figure 1. Clearly, neither drug significantly modified these metabolic consequences of coronary artery ligation, although the changes in coronary vein $O_2$ content, $Pco_2$ and pH were rather less than those seen in the control animals (Table 4).

Another metabolic change induced by coronary ligation is loss of potassium from ischaemic cardiac cells (Thomas, Shulman & Opie, 1970; Marshall & Parratt, 1975). In a series of untreated dogs, coronary vein potassium rose significantly from 3.5 ± 0.3 (pre-ligation) to 4.4 ± 0.2 mEq/litre, 30 min after ligation, although arterial and coronary sinus levels remained unchanged. In the dogs pretreated with MG 8926 the increase in coronary vein potassium (3.4 ± 0.2 to 3.9 ± 0.3 mEq/litre respectively) was significantly less than in the control animals. Similar results were obtained in the prenylamine-treated animals; coronary vein potassium rising from 3.0 ± 0.2 to 3.4 ± 0.2 mEq/litre ($P < 0.05$).

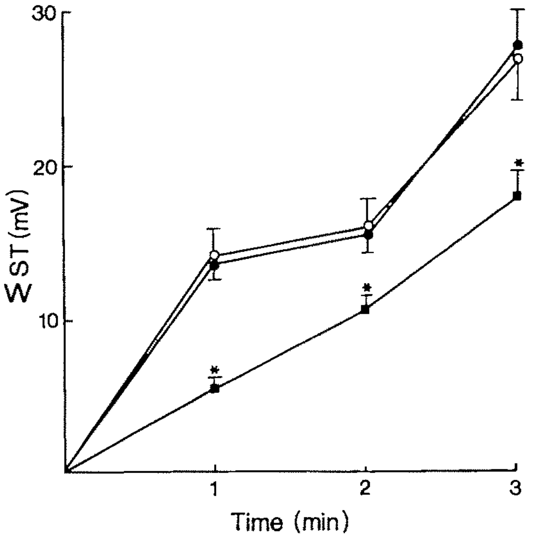


**Figure 2** Total ST-segment elevation in nine epicardial electrocardiographic leads at varying times after the occlusion of the anterior descending branch of the left coronary artery, 40 min before (●), 10 min before (○), and 10 min after (■) the intravenous administration of MG 8926 (5.0 mg/kg). Each point is the mean of five observations; vertical lines show s.e. mean. $^*P < 0.05$.

### *The haemodynamic effects of MG 8926 when administered intravenously, 1 to 2 h after acute coronary artery occlusion*

When administered 1 to 2 h after coronary artery ligation MG 8926 (5.0 mg/kg administered to 6 dogs) caused essentially similar effects to those seen before ligation. The drug caused an immediate, but transient, fall in blood pressure (mean diastolic change 63 ± 4 mmHg) and increased cardiac output (mean 34%) and both coronary sinus $Po_2$ (29 ± 3 to 56 ± 7 mmHg; $P < 0.05$) and coronary vein $Po_2$ (30 ± 1 to 49 ± 5

**Table 5** Haemodynamic changes induced by the intravenous injection of MG 8926 (5.0 mg/kg), 1 to 2 h after acute coronary artery ligation

| | *Pre-drug* | *10 min post-drug* |
|---|---|---|
| Heart rate (beats/min) | 170 ± 10 | 145 ± 8* |
| Systolic arterial blood pressure (mmHg) | 164 ± 9 | 167 ± 6 |
| Diastolic arterial blood pressure (mmHg) | 119 ± 6 | 123 ± 5 |
| Systolic pulmonary artery pressure (mmHg) | 17 ± 1 | 19 ± 1 |
| Diastolic pulmonary artery pressure (mmHg) | 8 ± 1 | 9 ± 1 |
| *LV* d*P*/dt *max* (mmHg/s) | 2140 ± 261 | 1870 ± 245* |
| LVEDP (mmHg) | 7 ± 2 | 12 ± 4* |
| Cardiac output (l/min) | 2.4 ± 0.3 | 2.6 ± 0.6 |
| External cardiac work (kgm/min) | 2.65 ± 0.21 | 3.40 ± 0.44 |
| Myocardial $O_2$ extraction (normal zone %) | 54 ± 6 | 50 ± 7 |
| Myocardial $O_2$ extraction (ischaemic zone – %) | 54 ± 4 | 52 ± 4 |

Values are mean ± s.e.; $n = 6$.
$^*P < 0.05$.

**Table 6** Comparison of the effects of saline, MG 8926 and prenylamine on ouabain-induced cardiac arrhythmias in the anaesthetized guinea-pig; the mean amounts of ouabain (μg/kg) required to produce the stated effects are given (±s.e.)

| | *Unequal intervals* | *Extrasystoles* | *Ventricular tachycardia* | *Ventricular fibrillation* | *Cardiac arrest* |
|---|---|---|---|---|---|
| Saline (control)<br>$n=7$ | 71± 4 | 92± 6 | 120±11 | 7/7<br>143±11 | 165±14 |
| MG 8926 1.0 mg/kg<br>$n=6$ | 90±11 | 113±15 | 153± 6* | 3/6<br>208±63* | 250±38* |
| Prenylamine 1.0 mg/kg<br>$n=6$ | 63± 7 | 94±10 | 130±16 | 3/6<br>168±35 | 169±24 |

*$P < 0.05$ compared with saline controls.

mmHg; $P < 0.05$). Although all these parameters had returned to control levels 10 min after injection, there were at this time significant decreases in heart rate and d$P$/d$t$ *max* and increases in LVEDP (Table 5).

### *The effect of intravenously administered MG 8926 on ST-segment changes of epicardial electrocardiograms*

The observation, in dogs treated with MG 8926, that the metabolic changes following coronary artery ligation were less marked than those seen in control animals prompted us to examine the effects of the drug on the extent and severity of myocardial ischaemia as assessed by epicardial electrocardiograms. The effects of prophylactically administered MG 8926 (5.0 mg/kg) on ST-segment elevation after acute occlusion of the LAD are shown in Figure 2. It is clear that, at this dose, MG 8926 decreases ST-segment elevation at all times after occlusion. In addition, the number of sites showing more than 2 mV ST-segment elevation, 3 min after occlusion, was significantly reduced (from 4.5 ± 0.6 to 2.5 ± 0.6; $P < 0.05$).

The effects of MG 8926 (5 mg/kg; five experiments) were also examined on ST-segment elevation in dogs in which the LAD had been ligated 1 to 2 h previously. The mean ST-segment elevation from all the seven sites varied in individual dogs (from 1.6 ± 0.9 to 5.1 ± 1.9 mV) but remained constant in each preparation over the 30 min preceding the injection of MG 8926. Particular attention was paid to the effect of MG 8926 on the border areas between normal and clearly ischaemic regions (usually leads 2, 3 and 4). In six such border sites, MG 8926 reduced mean ST-segment elevation from 4.3 ± 0.3 to 2.4 ± 0.6 mV ($P < 0.05$). This decrease in ST-segment elevation was still evident 20–30 min after administration of MG 8926. The results from one of these experiments are illustrated in Figure 3.

### *The effects of MG 8926 and prenylamine on ouabain-induced dysrhythmias in the anaesthetized guinea-pig*

The concentrations of ouabain required to produce (a) the first signs of rhythm abnormality (unequal R-R intervals), (b) ventricular extrasystoles, (c) sustained ventricular tachycardia, (d) ventricular fibrillation and (e) cardiac arrest in animals treated with either saline, MG 8926 (1 mg/kg) or prenylamine (1 mg/kg) are shown in Table 6. Injection of saline (<0.5 ml) had no significant effect on heart rate (320 ± 10 to 324 ± 9 beats/min, 30 s after injection). All control animals (saline-treated) developed ventricular fibrillation compared with only half of those treated with either MG 8926 or prenylamine. In addition MG 8926 (1 mg/kg) significantly increased the amount of ouabain necessary to induce ventricular tachycardia, ventricular fibrillation and cardiac arrest (Table 6). This dose of MG 8926 caused a significant decrease in

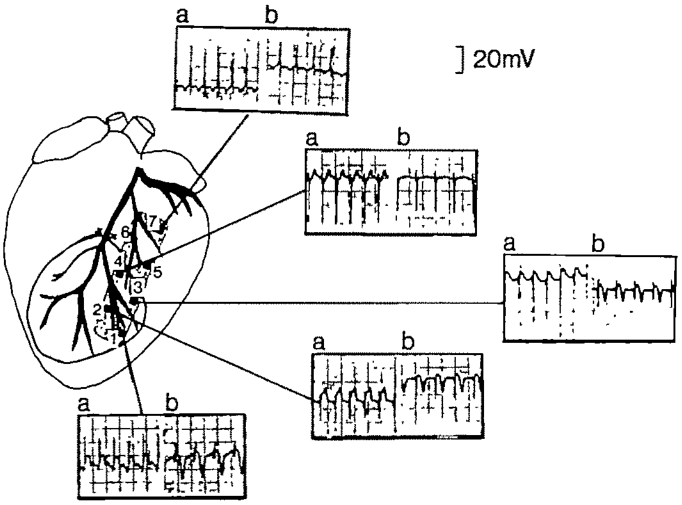


**Figure 3** Schematic diagram showing the siting of the epicardial leads in relation to the ischaemic portion of left ventricle produced by ligation of the anterior descending branch of the left coronary artery (X–X). Also shown are actual pairs of tracings from one dog (from leads 1, 2, 3, 4 & 7) taken immediately before (a) and 20 min after (b) injection of MG 8926 (5 mg/kg). Note that ST-segment elevation (indicative of ischaemia) is only present in leads 1, 2, 3 and 4 and that MG 8926 reduced the amount of ST-elevation in these leads.

heart rate from 353±9 to 317±9 beats/minute. In contrast, in animals treated with prenylamine (1 mg/kg intravenously), the amounts of ouabain needed to cause each type of dysrhythmia did not differ from those in the control animals. Prenylamine at this dose did not affect heart rate (312±16 beats/min before and 295±16 beats/min 5 min after injection). An attempt was made to investigate the effects of higher doses but both MG 8926 and prenylamine at an intravenous dose of 5 mg/kg caused such marked changes in the electrocardiogram that no investigation into possible antiarrhythmic effects at this dose level was possible. These toxic effects of both drugs were manifested first as severe bradycardia with T-wave reversal followed by conduction block and arrest.

### *The effects of MG 8926 and prenylamine on isolated electrically driven left atria of guinea-pigs*

Both MG 8926 (2.7–27 μM) and prenylamine (2.4–24 μM) caused dose-dependent decreases in developed tension of electrically driven guinea-pig atrial muscle. However at the lower concentrations this negative inotropic effect of both drugs was preceded by a transient increase in developed tension; dysrhythmias often occurred. A higher concentration of both drugs (>200 μM) caused sustained contracture.

Both MG 8926 and prenylamine significantly decreased the maximum driving frequency of atrial muscle. The magnitude of this effect was critically dependent on the time the drug was in contact with the tissue (Figure 4).

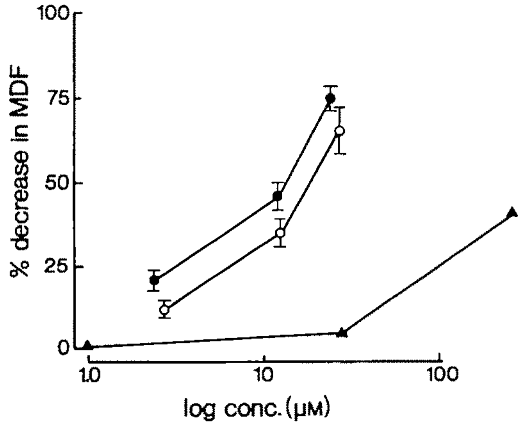


**Figure 4** The effects of MG 8926 (○) and prenylamine (●) on maximal driving frequency (MDF) of electrically driven guinea-pig atrial muscle. The ordinate scale represents percentage decrease in MDF and the abscissa scale is log concentration (μM). The contact time was 20 min except for (▲) which indicates effects of MG 8926 with contact time of 10 minutes. Note that the effect produced by MG 8926 is crucially dependent on this contact time.

## Discussion

Although previous experimental studies have demonstrated that prenylamine is capable of suppressing dysrhythmias induced either by hypothermia (Nielsen & Owman, 1967), or by the application of various chemicals (Lindner, 1963; Szekeres & Papp, 1971), there is no information available on the efficacy of prenylamine (or compounds, like MG 8926, of similar structure) in suppressing the early dysrhythmias that occur in the acute phase of experimental myocardial infarction. This information is of some importance since it is known that 61% of electrical deaths arising from myocardial infarction among patients younger than 65 occur within one hour of onset (Gordon & Kannel, 1971). The results obtained in this study have shown that both MG 8926 and prenylamine, when given intravenously in a dose of 5 mg/kg 20 min before ligation, protect anaesthetized greyhounds from the initial (30 min) burst of ventricular dysrhythmias which, in control dogs, are frequently fatal. For example, in this study, 4 out of 12 untreated dogs fibrillated within this crucial half hour period. None of the animals given MG 8926 and only one given prenylamine showed fibrillation during this period.

The protective effects of MG 8926 and prenylamine against ouabain-induced arrhythmias in anaesthetized guinea-pigs were much more equivocal. At a dose of 1 mg/kg only MG 8926 significantly increased the amount of ouabain required to cause ventricular tachycardia, ventricular fibrillation and cardiac arrest although both compounds reduced the incidence of fibrillation induced by the glycoside. The reasons for these equivocal findings are not immediately apparent but of possible relevance are the findings of Lindner (1963) who showed, in anaesthetized guinea-pigs, that although prenylamine (2–13 mg/kg, intravenously) protected against the arrhythmias induced by digoxin, no protection was afforded when ouabain was used to induce arrhythmias.

In theory, there are several possible explanations for the anti-arrhythmic properties shown by prenylamine and related compounds. First, prenylamine is known to cause a partial depletion of catecholamine stores in cardiac adrenergic nerves (Schone & Lindner, 1962). This effectively reduces cardiac sympathetic drive which has been implicated in the genesis of early arrhythmias induced either by coronary ligation (Harris, Estandia & Tillotson, 1951; Harris & Bisteni, 1955; Shanks & Dunlop, 1967) or by infusion of cardiac glycosides (Dohadwalla *et al.*, 1969). Secondly, prenylamine causes direct effects on various phases of the cardiac action potential. Thus it has been shown that prenylamine slows the maximum rate of rise (phase 0), increases the threshold potential, increases refractory period and depresses pacemaker activity (Lindner, 1963). These direct effects of prenylamine are now thought to be due to depression of the inward sodium and/or calcium currents involved in cell depolarization processes (Haas, Kern, Benninger & Einwachter, 1975). It would seem probable, but has not yet been established, that MG 8926 possesses at least some of these properties of prenylamine. Certainly in this study, MG 8926 increased the effective refractory period of electrically-driven guinea-pig atrial muscle, in concentrations similar to those needed with prenylamine.

At dose levels which were clearly antidysrhythmic, both MG 8926 and prenylamine caused only transient haemodynamic changes. The major immediate effects common to both drugs were decreases in blood pressure and increases in cardiac output and coronary blood flow. Since $O_2$ extraction was considerably reduced, neither drug changed myocardial $O_2$ consumption. The vasodilatation induced by MG 8926 and prenylamine may be due, like that seen with verapamil, to inhibition of the depolarization-induced calcium influx in the smooth muscle of the peripheral and the coronary arteries (Peiper, Griebel & Wende, 1971). The transient but considerable increases in cardiac output caused by both drugs may also be due to an effect on cardiac calcium flux.

It has been demonstrated in anaesthetized cats, and rat isolated perfused hearts, that prenylamine is capable of releasing substantial amounts of noradrenaline (Grobecker, Palm & Holtz, 1968). In this present investigation, further circumstantial evidence that prenylamine causes noradrenaline release is available from the fact that, immediately on injection, it increased heart rate and myocardial contractility (as measured by d*P*/d*t max*) and evoked marked ventricular ectopic activity. Interestingly, MG 8926 did not cause positive chronotropic or inotropic effects *in vivo*, nor did it induce ectopic activity.

On guinea-pig isolated atrial muscle, both compounds (2–30 μM) decreased developed tension, in a dose-dependent manner. This was usually preceded by a transient positive inotropic effect. These results confirm previous findings with prenylamine (Fleckenstein, Kammermeier, Döring & Freund, 1967; Lindner, 1969). The view has recently been presented that this inotropic effect of prenylamine may be due to an inhibition of active calcium transport within the cell, leading to transient increases in available calcium (Haas *et al.*, 1975).

It has been shown that, by means of epicardial ECG mapping of the ischaemic area of cardiac muscle, that it is possible to evaluate the extent and severity of damage caused by coronary artery ligation (Maroko, Kjekshus, Sobel, Watanabe, Covell, Ross & Braunwald, 1971; Wendt, Canavan & Michalak, 1974). With this technique it has recently been demonstrated that verapamil, a calcium antagonist drug, is capable of reducing both the extent and severity of ischaemia induced by ligation of a coronary artery (Smith, Singh, Nisbet & Norris, 1975; Wende, Bleifeld, Meyer & Stühlen, 1975). In the present investigation there was some evidence that MG 8926 (administered either before or after occlusion) was capable of favourably influencing the balance between myocardial $O_2$ supply and demand (as assessed by epicardial ST-segment changes) especially in border areas surrounding the developing infarct. The mechanism behind this reduction in ischaemia seen after MG 8926 may involve both bradycardia and a reduction in the resistance to blood flow in the ischaemic zone. It has certainly been established that a decrease in heart rate is important for the beneficial effects of $\beta$-adrenoceptor antagonists in myocardial ischaemia (Gross & Winbury, 1973; Marshall & Parratt, 1976) and that propranolol decreases the degree of ischaemia-induced ST-segment elevation largely by slowing heart rate (Wendt *et al.*, 1974). In this study it was observed that MG 8926 only reduced heart rate when administered after coronary artery occlusion, a fact which may be related to the effects of the drug on the increased sympatho-adrenal outflow produced by myocardial ischaemia (Staszewska-Barczak & Ceremuzynski, 1968).

We wish to thank Dr Ralph Kohn (Advisory Services, London) for making available to us generous supplies of MG 8926. The dog experiments were carried out in the Department of Surgery (University of Glasgow) through the kindness of Dr Iain McA. Ledingham.

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*(Received June 22, 1976.)*

*Br. J. Pharmac.* (1977), 59, 323–325

# THE EFFECT OF (—)-ISOPRENALINE AND (±)-SALBUTAMOL ON PEPSINOGEN AND ACID SECRETION IN THE DOG

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1 The β-adrenoceptor agonists, (—)-isoprenaline and (±)-salbutamol, reduced pepsinogen secretion induced by pentagastrin in conscious dogs with Heidenhain pouches.

2 (—)-Isoprenaline and (±)-salbutamol also reduced gastric acid secretion while producing a moderate tachycardia.

## Introduction

β-Adrenoceptor agonists inhibit gastric acid secretion induced by pentagastrin and food in the dog (Curwain & Holton, 1972) and man (Fielding, Kilborn & Russell, 1975). However, little is known about the effects of β-adrenoceptor agonists on pepsinogen secretion. Holton (1973), reviewing the available information, concluded that catecholamines probably stimulate pepsinogen secretion. We wish to report studies in which the β-adrenoceptor agonists (—)-isoprenaline and (±)-salbutamol inhibited pepsinogen secretion induced by pentagastrin in conscious dogs with vagally denervated (Heidenhain) pouches.

## Methods

Male beagle dogs (13.3–15 kg) with well-established Heidenhain pouches were used. Food was withheld but water allowed for 18 h before each experiment. The dogs stood in slings during the experiment. A sterile catheter was inserted into a superficial limb vein and an infusion of sterile saline (0.9% w/v NaCl solution) given at the rate of 1 ml/minute. All drugs were administered in this saline infusion.

Submaximal gastric secretion was induced by a continuous infusion of pentagastrin. In each dog a dose of pentagastrin which would give a 50% maximal secretory response was determined as follows. Pentagastrin was infused at 0.5 μg $kg^{-1}$ $h^{-1}$ for 1 h or until a plateau of gastric acid secretion was obtained. The dose of pentagastrin was then doubled, and this dose infused until a new plateau of secretion was reached. The procedure was repeated until doubling the dose of pentagastrin produced no further increase in gastric acid secretion. Secretory responses were calculated as percentage of maximum response to pentagastrin and the dose required to elicit 50% of maximum secretion was determined for each dog. These pentagastrin doses, which were used for all further experiments, were 1, 4 and 4 μg $kg^{-1}$ $h^{-1}$ for dogs 1, 2 and 3 respectively.

The pouch secretion was allowed to drain into a collection vessel which was changed every 15 minutes. The volume of secretion was measured to the nearest 0.1 ml and an aliquot titrated against 0.1 mol/l NaOH to pH 7 using a Radiometer TTT2 titration system. Acid output was calculated in μmol $H^+$/minute. The remaining sample was deep frozen for subsequent determination of pepsin content by a modification of the autoanalyser method of Vatier, Cheret & Bonfils (1968). Pepsin content is expressed in pepsin units where 1 pepsin unit is the enzymatic activity required to release 1 μmol of tyrosine per min from 4% ox haemoglobin substrate at pH 2.0 and 37°C. Heart rate was measured at 15 min intervals by palpation.

Once a plateau of gastric acid secretion had been obtained (less than 10% variation over 1 h), (—)-isoprenaline 1 or 3 ng $kg^{-1}$ $min^{-1}$ or (±)-salbutamol 30 or 100 ng $kg^{-1}$ $min^{-1}$ was infused concurrently with the pentagastrin for 1 hour. Inhibition of secretion started in the first 15 min collection period and achieved equilibrium during the last 30 min of the infusion. Infusion of pentagastrin alone was then continued until the secretory response returned to the control level. Only one dose level of β-adrenoceptor agonist was administered in each experiment and at least 5 days elapsed between experiments.

Results have been calculated as % change in the measured parameters, by comparing the mean of the two consecutive values at peak drug response with the mean of the four control values preceding the drug infusion.

The drugs used were (—)-isoprenaline bitartrate dihydrate (Ward, Blenkinsop & Co. Ltd.), (±)-

salbutamol (Allen & Hanburys Ltd.) and pentagastrin (ICI Ltd.). Drug doses are expressed as base. All solutions of (–)-isoprenaline contained ascorbic acid 20 µg/ml.

## Results

In each dog the control values for volume (secretion rate ml/min) and acid (secretion rate µmol $H^+$/min) secreted by the Heidenhain pouch submaximally stimulated by pentagastrin were consistent from experiment to experiment. The concentration of pepsin in units/l and in consequence the rate of pepsin secretion in units/min was more variable. Control heart rates were consistent for each dog. The mean control values ± standard error ($n$=number of experiments) for dog 1 were as follows: acid secreted 61.6±3.0 µmol $H^+$/min, volume secreted 0.42±0.02 ml/min, pepsin concentration 86.6±26.8 units/l, pepsin secretion rate 23.3±7.4 units $\times 10^{-3}$/min and heart rate 68.6±2.7 beats/min ($n$=11). For dog 2 corresponding values were, 43.8±3.4, 0.30±0.02, 136.4±39.5, 45.7±14.0 and 65.4±3.1 ($n$=9). For dog 3 corresponding values were, 14.7±1.3, 0.12±0.01, 123.9±21.4, 13.7±2.3 and 76.2±2.7 ($n$=11). The secretory outputs for each dog were approximately 50% of their maximum response to pentagastrin and the magnitude of the response probably reflects the pouch size.

The results summarized in Table 1 show that (–)-isoprenaline (1 and 3 ng $kg^{-1}$ $min^{-1}$) and (±)-salbutamol (30 and 100 ng $kg^{-1}$ $min^{-1}$) infusion over 1 h reduced the volume of gastric secretion, the rate of acid and pepsinogen secretion in a dose-related manner. The respective $ED_{50}$ values in ng/kg (95% confidence limits) for (–)-isoprenaline were 2.1 (1.8–2.6), 2.3 (2.0–2.7) and 1.9 (1.5–2.7). The corresponding values for (±)-salbutamol were 56.1 (24–104), 62.0 (20–233) and 53.9 (19–164).

The concentration of pepsinogen was not significantly changed by (–)-isoprenaline or (±)-salbutamol and $ED_{50}$ values were not calculated. The concentration of acid in the secretion did not alter significantly in response to (–)-isoprenaline or (±)-salbutamol administration.

(–)-Isoprenaline and (±)-salbutamol caused dose-related increases in heart rate, with a maximum increase of 76 and 107 beats/min respectively at the highest dose levels. All responses to (–)-isoprenaline showed complete recovery within 15–30 min and those to (±)-salbutamol within 45–180 min of the end of the drug infusion.

## Discussion

In the present study using conscious dogs with Heidenhain pouches, intravenous infusion of total doses of 0.06 and 0.18 µg/kg (–)-isoprenaline or 1.8 and 6 µg/kg (±)-salbutamol inhibited pepsinogen secretion. These doses also produced tachycardia and are in the dose range eliciting $\beta$-adrenoceptor mediated responses (Daly, Farmer & Levy, 1971; Daly, Flook & Levy, 1975). The potency of (±)-salbutamol relative to (–)-isoprenaline as an inhibitor of pepsinogen secretion was 28 compared with values of 6 to 25 for $\beta_2$-adrenoceptor systems in the dog (Daly *et al.*, 1971) suggesting that the observed inhibition of pepsinogen secretion is mediated through $\beta_2$-adrenoceptors. Attempts to antagonize the effects of (–)-isoprenaline on gastric secretion with propranolol yielded equivocal results due to potentiation of pentagastrin-induced secretion by propranolol (Lin & Evans, 1973; Daly & Stables, unpublished results).

The inhibition of pentagastrin-induced gastric acid secretion by (–)-isoprenaline and (±)-salbutamol confirms other studies with (±)-isoprenaline (Curwain & Holton, 1972) and (±)-salbutamol (Curwain, Holton & Spencer, 1972). It has been proposed that $\beta$-adrenoceptor agonists reduce pentagastrin-stimulated

**Table 1** Effects of (–)-isoprenaline and (±)-salbutamol on pentagastrin-stimulated gastric secretion in the conscious Heidenhain pouch dog

| | | *Mean % change ± standard error* | | | | |
|---|---|---|---|---|---|---|
| *Drug* | *Dose ng $kg^{-1}$ $min^{-1}$ for 60 min* | *Acid** | *Volume** | *Pepsinogen* concentration* | *Pepsinogen* secretion rate* | n |
| (–)-Isoprenaline | 1 | –23.3±4.6 | –18.9±2.9 | –7.6±29.1 | –23.5±9.7 | 10 |
| | 3 | –62.9±4.2 | –59.9±3.9 | –29.1±8.8 | –67.6±3.3 | 11 |
| (±)-Salbutamol | 30 | –33.8±8.4 | –34.6±7.8 | 0.0±17.4 | –37.8±11.8 | 5 |
| | 100 | –58.5±8.8 | –55.1±7.7 | 19.9±15.5 | –60.9±7.8 | 7 |

* units as defined in methods
$n$=number of experiments.

acid secretion by inhibiting histamine formation or release (Curwain, Holton, McIsaac & Spencer, 1974; Lundell & Svensson, 1974). However, the site and mechanism of action by which (−)-isoprenaline and (±)-salbutamol inhibit pepsinogen secretion are unknown and their elucidation requires further investigation.

The authors wish to thank Mr C. Harrison for carrying out the pepsin assays.

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*(Received June 28, 1976.*
*Revised September 10, 1976.)*

*Br. J. Pharmac.* (1977), 59, 327–332

# A POSSIBLE ROLE FOR CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE IN THE REGULATION OF ACID SECRETION IN THE ISOLATED STOMACH OF GUINEA-PIG

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**1** The rate of acid secretion and mucosal cyclic adenosine 3′,5′-monophosphate (cyclic AMP) content have been measured on the same guinea-pig isolated stomach preparation in response to histamine, theophylline and ICI 63197, a potent phosphodiesterase inhibitor.

**2** Unstimulated control tissues had a spontaneous rate of acid secretion of $74.41 \pm 9.06$ μmol $H^+$/g wet wt. of mucosa per hour (s.e. mean, $n=20$) and a cyclic AMP content of $0.517 \pm 0.058$ nmol/g wet weight.

**3** Each of the three drugs caused an increase in both the mucosal cyclic AMP content and the rate of acid secretion. These increases were linearly related to the logarithm of drug concentration for each drug.

**4** There were no statistically significant differences between the three regression coefficients obtained for acid on drug and for cyclic AMP on drug.

**5** There was a significant correlation between the rate of acid secretion and mucosal cyclic AMP content in stimulated preparations ($P < 0.001$) and also in control preparations which received no drug ($P < 0.05$).

**6** These results are discussed in relation to the possible role of cyclic AMP in the mediation of acid secretory responses in the mammalian stomach.

## Introduction

Cyclic adenosine 3′,5′-monophosphate (cyclic AMP) has been implicated as the intracellular mediator of a diverse range of physiological responses. Its possible role as a mediator of acid secretion in the mammalian stomach has been recently reviewed (Amer, 1972; 1974; Kimberg, 1974). Amer concluded that increased cyclic AMP in the stomach was associated with a decrease in acid secretion. He explained the stimulatory effects of phosphodiesterase inhibitors in terms of changes in the microcirculation to the mucosa and alterations in the calcium ion permeability of the cells. However, Kimberg concluded that, in some mammalian species at least, acid secretion was associated with an increase in cyclic AMP. More recent publications (Ruoff & Sewing, 1974; 1975; Katsumata & Glick, 1975) have added further support to the hypothesis that increased acid secretion is associated with increased cyclic AMP in the rat stomach. If acid secretion is regulated by the cyclic AMP content of the mucosa one would expect there to be a quantitative relationship between mucosal cyclic AMP and the rate of acid secretion. This paper describes experiments in which the rate of acid secretion and the mucosal cyclic AMP content have been measured on the same guinea-pig isolated stomach preparation. The use of an *in vitro* preparation has the advantage that the results are not complicated by changes in either mucosal blood flow or circulating hormone levels which might occur in an intact animal. Some preliminary results have been communicated to the Physiological Society (Canfield, Curwain & Spencer, 1976).

## Methods

The preparation and general procedures are those described previously by Holton & Spencer (1976). The stomach was divided into two halves along the greater curvature and each half was tied over the end of a short perspex tube, mucosal surface facing the lumen. The muscle coat was not removed. The serosal surface was bathed with a bicarbonate buffered saline and gassed with 95% $O_2$ and 5% $CO_2$ whilst the mucosal surface was bathed with a similar but unbuffered saline gassed with 100% $O_2$. In the current series of experiments the volume of serosal bathing fluid was 30 ml and the rate of acid secretion was determined by titration with 20 mM NaOH. Following dissection, the preparations were set up in the tissue bath and gassed for 1 h before addition of drugs. The mucosal saline was changed every 15 min and the rate of spontaneous acid secretion determined. After the addition of drugs, acid secretion was measured for four further 15 min periods. The rate associated with the drug was taken as the average of the last two such periods. Tissues were then taken from the bath and placed in ice cold saline before extraction for cyclic AMP assay.

### *Tissue extraction and cyclic AMP assay*

Each tissue was pinned out on a board that had been cooled to −18°C. The tissue was blotted to remove surface moisture and the mucosal layer quickly scraped off, transferred to a glass cover slip and kept at 4°C. The scraped mucosa was quickly weighed and transferred to a plastic centrifuge tube in an ice-water bath. The remaining procedures were carried out at 0°C. The tissue was homogenized in the tube with 0.5 ml of 0.5 M perchloric acid and then neutralized with 0.5 ml of 0.5 M $K_2CO_3$. The mixture was centrifuged for 30 min at 2000 *g* at 0°C; 0.5 ml of the supernatant was added to another tube containing 0.1 ml of cold 0.5 M Tris/HCl buffer, pH 7.5, and stored at −18°C until assayed. Cyclic AMP was usually measured within two days by an isotope dilution technique. This was obtained in kit form and had a limit of detection of 0.05 pmol/50 µl of sample. The coefficient of variation for repeated measurements given by manufacturers is less than 7% over the range 0.5–14 pmol/50 µl sample. (The cyclic AMP assay. Radiochemical Centre, Amersham, 1975). Tritium was counted on a Hewlett-Packard Tricarb liquid scintillation counter using a methanol/toluene scintillator.

### *Drugs and chemicals*

The following were used as stimulants; theophylline hydrate (BDH), 2 amino-6-methyl-5-oxo-4-*n*-propyl-4,5-didhydro-*s*-triazolo (1,5-a) pyrimidine (ICI 63197), histamine acid phosphate (1 mg/ml; Macarthys) and dibutyryl cyclic AMP (Sigma). These were always added to the serosal bathing fluid.

### *Experimental design*

The tissue bath accomodated 6 half-stomach preparations from 3 animals. Two were used as controls (O), two for one drug dose (A) and two for another (B). This provided three 'dose pairs'; (OA), (OB) and (AB) which were randomly allocated to the 3 animals. The allocation of the dose pair within the half-stomach pair was also randomized.

This design was adopted to facilitate a comparison between the two half-stomachs from the same animal. However, examination of the results indicated that the within animal variance was not any smaller than that between animals. Consequently, each half-stomach has been treated as an individual observation for the purposes of statistical analysis.

In a few experiments, accurate estimates of the acid secretory rate were not obtained. This was due to leakage of bicarbonate from the serosal bathing fluid resulting from physical damage to the preparation by the tube used to remove the mucosal bathing fluid.

The rate of acid secretion is expressed as µmol $H^+$/g wet wt. mucosa per h and the cyclic AMP content as nmol/g wet wt. of mucosa.

## Results

### *Unstimulated control tissues*

All the tissues secreted acid spontaneously, the mean value for controls during the second hour was 74.41 ± 9.06 µmol $H^+ g^{-1} h^{-1}$ (s.e. mean, $n = 20$). This was equivalent to 2.79 ± 0.63 µmol $H^+ cm^{-2} h^{-1}$. The mean cyclic AMP content of these tissues was 0.517 ± 0.058 nmol/g (s.e. mean, $n = 20$), comparable to the values found by Karppanen, Neuvonen, Bieck & Westermann (1974) using isolated mucosa from the guinea-pig.

### *Phosphodiesterase inhibitors*

Figure 1a shows graphs of the rate of acid secretion as a function of the logarithm of drug concentration for the two drugs, theophylline and ICI 63197 and Figure 1b shows similar graphs for the mucosal cyclic AMP content. There are more values for cyclic AMP than acid because accurate estimates of acid secretion were not obtained from some tissues for reasons described in the Methods section. The inclusion of those 'extra' points for cyclic AMP is justified when one is estimating the effects of drug concentrations on a single parameter. Both the rate of acid secretion and cyclic AMP content were linearly related to the logarithm of the drug concentration over the range used for both drugs ($P < 0.05$ in all four cases). The values of the regression coefficients of acid on drug concentration were not significantly different for the two drugs, nor were those for cyclic AMP on drug concentration. However, ICI 63197 is more potent than theophylline on a molar basis.

### *Reversal of the effect of theophylline*

The effect of theophylline on the rate of acid secretion is readily reversible (Holton & Spencer, 1976). Table 1 shows the results of experiments in which all

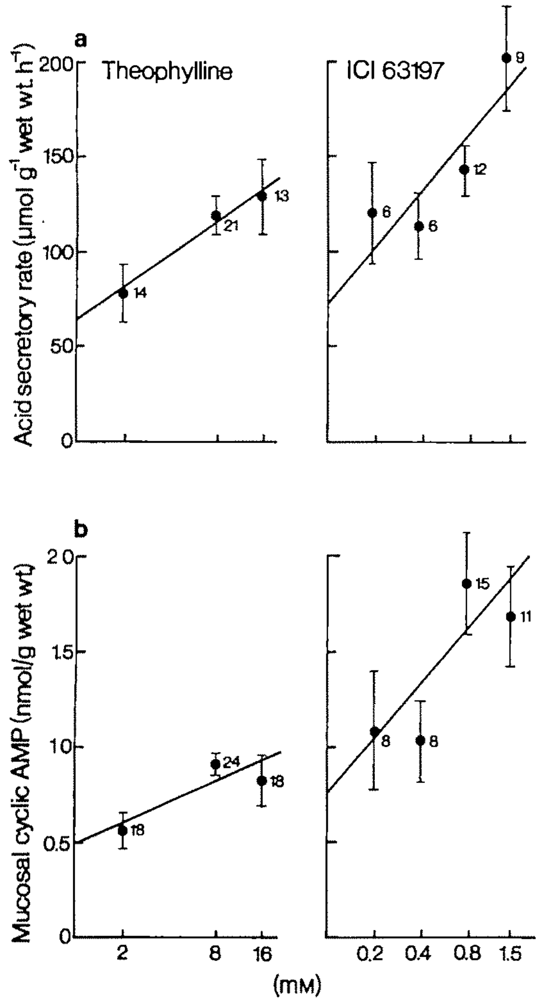

**Figure 1** The relationships between the concentrations of phosphodiesterase inhibitors (theophylline or ICI 63197) plotted on a logarithmic scale and (a) the rate of acid secretion (μmol $H^+$ $g^{-1}$ $h^{-1}$) and (b) mucosal cyclic adenosine 3',5'-monophosphate (cyclic AMP) content (nmol/g) in the guinea-pig isolated stomach. Vertical lines show s.e. means. Number of preparations is given beside each point.

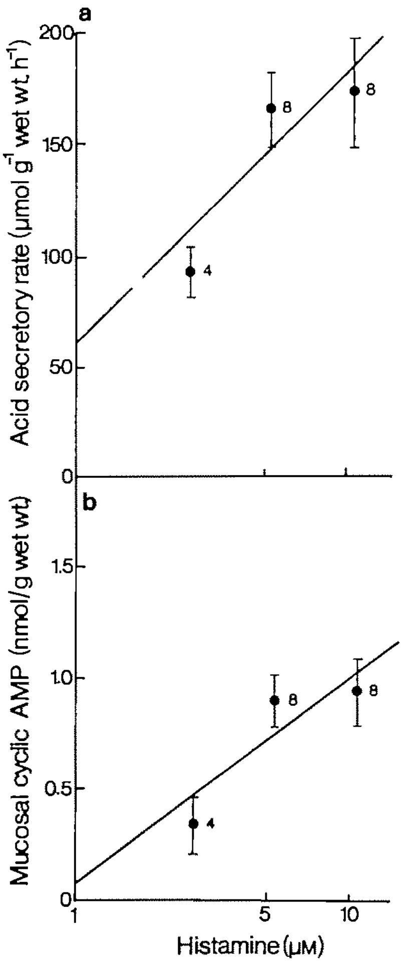

**Figure 2** The relationship between the concentration of histamine plotted on a logarithmic scale and (a) the rate of acid secretion (μmol $H^+$ $g^{-1}$ $h^{-1}$) and (b) mucosal cyclic adenosine 3',5'-monophosphate (cyclic AMP) content (nmol/g) in the guinea-pig isolated stomach. Vertical lines show s.e. means. Number of preparations is given beside each point.

tissues received 8 mM theophylline during the first hour of the experiment. This was withdrawn from one member of each pair during the second hour. The tissues were then extracted for cyclic AMP assay as before. During the first hour, the mean rate of acid secretion for the two groups (2 h drug and 1 h drug + 1 h recovery) were not significantly different but there was a significant difference ($P < 0.02$) during the second hour as expected. The recovery group had a significantly ($P < 0.02$) lower cyclic AMP content than the group continuously exposed to the drug. The values for the rate of acid production and cyclic AMP content for the group from which the theophylline was removed were not significantly different from those described above under control tissues.

### *Histamine*

Figure 2 shows the relation between the rate of acid production and the logarithm of histamine concentration and cyclic AMP content and the logarithm of histamine concentration. Both parameters were linearly related to the logarithm of drug concentration and the regression coefficients for acid on histamine and cyclic AMP on histamine were not significantly different from those obtained with the phosphodiesterase inhibitors. This is summarized in Table 2.

### *Dibutyryl cyclic AMP*

The rate of acid secretion of 5 preparations was increased by an average of 25% following application of 1 mM dibutyryl cyclic AMP. This increase was not statistically significant. Addition of 1 mM dibutyryl cyclic AMP during a plateau response to 1 mM theophylline caused a further 50% increase in the rate of acid secretion above the theophylline plateau. This increase was significant ($P < 0.01$).

### *Relationship between mucosal cyclic AMP and rate of acid secretion*

An analysis of covariance between cyclic AMP and acid secretion was performed on the paired values from the same tissue, pooling the results for all three drugs. A correlation coefficient ($r$) of 0.3944 with 96 degrees of freedom was obtained indicating a very significant correlation between them ($P < 0.001$). A significant correlation ($r = 0.4828$, d.f. = 18, $P < 0.05$) was also obtained when the paired values from control tissues which had not received any drug were analysed. The linear regression coefficient of the rate of acid secretion on the cyclic AMP content of the mucosa obtained from the covariance analysis of the control data did not differ significantly from that obtained from the analysis of the stimulated tissues.

## Discussion

Interpretation of these results is complicated by the fact that the gastric mucosa is a heterogeneous tissue. The extraction technique used in this work does not selectively measure the cyclic AMP content of the parietal cells which are responsible for acid secretion. Katsumata & Glick (1975) have shown that histamine causes an increase in the cyclic AMP content only in the parietal–mucus neck cell layer of the rat gastric mucosa whilst theophylline has a similar effect and also causes a smaller increase in the cyclic AMP content of the chief cell layer. As there are no statistically significant differences between our results for histamine and theophylline we feel it is reasonable to assume that most of the changes in cyclic AMP we have observed have taken place in the parietal–mucus neck cell layer but must bear in mind possible species differences between rat and guinea-pig. The action of theophylline and histamine on acid secretion confirm earlier findings (Holton & Spencer, 1976) whilst the results with ICI 63197 contrast with those of Wan (1976) who found that this drug caused only a slight stimulation of acid secretion in the mouse isolated

**Table 1** Recovery of the rate of acid secretion and mucosal cyclic adenosine 3′,5′-monophosphate (cyclic AMP) to control levels following treatment with 8 mM theophylline for one hour

| | *Drug for 2 h* | | | *Drug 1 h+1 h recovery* | | |
|---|---|---|---|---|---|---|
| | *Acid secretion ($\mu$mol H$^+$ g$^{-1}$ h$^{-1}$)* | | *cyclic AMP (nmol/g)* | *Acid secretion ($\mu$mol H$^+$ g$^{-1}$ h$^{-1}$)* | | *cyclic AMP (nmol/g)* |
| | *1st h* | *2nd h* | | *1st h* | *2nd h* | |
| Mean | 129.3 | 109.6 | 0.858 | 118.4 | 77.6 | 0.542 |
| s.e. mean | 13.7 | 9.7 | 0.061 | 13.5 | 9.1 | 0.066 |
| *n* | 19 | 19 | 20 | 19 | 18 | 18 |

**Table 2** Summary of the linear regression data for the rate of acid secretion and cyclic adenosine 3′,5′-monophosphate (cyclic AMP) content on the logarithm of drug concentration

| | | *Rate of acid secretion* | | | *cyclic AMP content* | |
|---|---|---|---|---|---|---|
| *Drug* | n | *Correlation coefficient* | *Regression coefficient* | n | *Correlation coefficient* | *Regression coefficient* |
| Theophylline | 48 | 0.3630 | 58.56 | 47 | 0.3362 | 375.00 |
| ICI 63197 | 33 | 0.4519 | 96.71 | 41 | 0.3396 | 963.63 |
| Histamine | 20 | 0.4664 | 117.41 | 20 | 0.5259 | 873.35 |

stomach but did potentiate the action of histamine and pentagastrin. The effect of histamine on the guinea-pig mucosal cyclic AMP content is similar to the results of Domschke, Domschke, Classen & Demling (1973) obtained from the intact rat.

The phosphodiesterase inhibitors might be stimulating acid secretion via a mechanism involving calcium as suggested by Amer (1972) with a concentration-dependent increase in cyclic AMP as a side effect. However, there is no evidence that histamine changes calcium permeability but it has been reported to stimulate adenyl cyclase activity in the gastric mucosa of rat and rabbit. (Bersimbaev, Argutinskaya & Salganik, 1971; Sung, Jenkins, Burns, Hackney, Spenney, Sachs & Wiebelhaus, 1973). Thus, an increase in the mucosal cyclic AMP content brought about by two different means, phosphodiesterase inhibition and adenyl cyclase activation, is associated with an increase in the rate of acid secretion in our experiments. Both the rate of acid secretion and the mucosal cyclic AMP content increased linearly with the logarithm of the drug concentration for all three drugs and the respective linear regression coefficients were not significantly different between the drugs (see Table 2). This does not mean that all three drugs are acting on a common mechanism nor does it prove a causal relationship between cyclic AMP content and the rate of acid secretion. However, it does suggest that there is no reason to treat the results from the three drugs seaparately and they were pooled for the purpose of the analysis of covariance. Again a significant correlation is not proof of a causal relationship between the parameters. We cannot, for example, completely exclude the possibility that the cyclic AMP content of the mucosa is affected by the concentration of acid in the mucosal bathing fluid which is a function of the drug concentration.

The non-significant increase in acid secretion in response to exogenous dibutyryl cyclic AMP in this preparation contrasts with findings on the rat (Brennan, Arbakov, Stefankiewicz & Groves, 1975) and rabbit (Fromm, Schwartz & Quijano, 1975) stomachs, but this may be due to a species difference in the metabolism of cyclic nucleotides. The potentiating effect of theophylline suggests that the rate of entry of dibutryryl cyclic AMP in this preparation may be so low that it is largely destroyed by the phosphodiesterase and a significant increase in acid secretion is only obtained when there is partial inhibition of this enzyme. However, Main & Whittle (1974) have shown that the stimulatory effect of dibutyryl cyclic AMP in the rat is potentiated by a threshold dose of histamine. We have not tested this with the guinea-pig preparation but it is of interest that both theophylline and histamine stimulation are associated with an increase in mucosal cyclic AMP content. It may be that the action of exogenous dibutyryl cyclic AMP is potentiated in some way by a simultaneous increase in endogenous cyclic AMP.

Our results indicate that acid secretion in control preparations is related to the mucosal cyclic AMP content. The stimulatory action of histamine and phosphodiesterase inhibitors may be due to their action in increasing the cyclic AMP content. This provides further evidence to support the hypothesis that cyclic AMP could be one of the factors in the regulation of acid secretion.

We would like to thank St. Mary's Hospital Joint Standing Research Committee and the University of London Central Research Fund for support and Dr A.H. Todd of ICI Ltd for the gift of ICI 63197.

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*(Received June 28, 1976.
Revised August 27, 1976.)*

*Br. J. Pharmac.* (1977), 59, 333–341

# MODIFICATION OF CARDIOVASCULAR RESPONSES TO HISTAMINE BY DITHIOTHREITOL

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1 Histamine produced dose-dependent contractile responses on both isolated perfused ear arteries and aortic strips of the rabbit. These responses were blocked by mepyramine and potentiated by both metiamide and dithiothreitol.

2 In the presence of maximum potentiation by metiamide, dithiothreitol still potentiated the contractile response to histamine of both preparations.

3 In the presence of mepyramine, histamine produced dose-dependent reductions in the contractile response to noradrenaline. This vasodilator action of histamine was abolished by metiamide but was unaffected by dithiothreitol.

4 The vasodilator action of histamine on the human isolated perfused temporal artery and the positive inotropic effect of histamine on the isolated spontaneously beating atria of the rabbit were blocked by metiamide but unaffected by dithiothreitol.

5 It is concluded that the rabbit aorta, like the ear artery, contains both $H_1$ and $H_2$ histamine receptors and that dithiothreitol potentiates cardiovascular responses mediated by $H_1$-receptors but not by $H_2$-receptors.

## Introduction

Finding that dithiothreitol (DTT, Cleland's reagent), an agent which reduces disulphide linkages to sulphydryl groups, potentiates the constrictor response of the rabbit aorta to histamine, Fleisch, Krzan & Titus (1973) concluded that disulphide bridges occupied prominent positions either in histamine receptors or along the excitation-contraction pathway.

However, it is known that responses to histamine are mediated by two types of receptor (Black, Duncan, Durant, Ganellin & Parsons, 1972). In the rabbit, the biphasic effect which histamine has on arterial pressue is a balance between a pressor effect mediated by $H_1$-receptors and a depressor effect mediated by $H_2$-receptors (Parsons & Owen, 1973; Carroll, Glover & Latt, 1974). Further, both $H_1$ and $H_2$-receptors have been demonstrated in the same isolated artery; thus the response of the rabbit ear artery is a balance between a predominant constrictor action mediated by $H_1$-receptors and a vasodilator action mediated by $H_2$-receptors (Glover, Carroll & Latt, 1973; Parsons & Owen, 1973).

The present experiments were therefore designed to study the actions of DTT on cardiovascular responses mediated by both types of receptor. A brief account of some of these results has been published (Carroll & Glover, 1974).

## Methods

Isolated segments of rabbit ear and human temporal arteries were cannulated, and perfused at constant flow with Krebs bicarbonate solution of the following composition (mmol/l): NaCl 118, $NaHCO_3$ 25, $NaH_2PO_4$ 1.33, KCl 4.7, $CaCl_2$ 2.7, $MgCl_2$ 1.44 and glucose 5.5, aerated with 5% $CO_2$ in $O_2$. Human temporal arteries were obtained at autopsy within 6–12 h after death. The flow rate through the arteries was kept constant in the range 5–10 ml/min, so perfusion pressures in the absence of any drugs were 20–30 mmHg for the rabbit ear and 10–40 mmHg for the human temporal vessels. Care was taken to ensure that the cannula contributed negligible resistance. Perfusion pressure was measured with a Statham P23AC pressure transducer and recorded on a Grass polygraph. Drugs were injected in volumes of 0.05–0.2 ml through a rubber connection close to the cannula or added directly to the perfusate.

Helically cut segments of rabbit aorta were suspended in a 15 ml organ bath at an initial tension of 1 gram. Krebs bicarbonate solution was continually pumped through the bath at a rate of 25 ml/min and drugs were either injected in volumes of 0.05–0.5 ml into the perfusion stream or added directly to the perfusate. Contractions were measured with a Grass

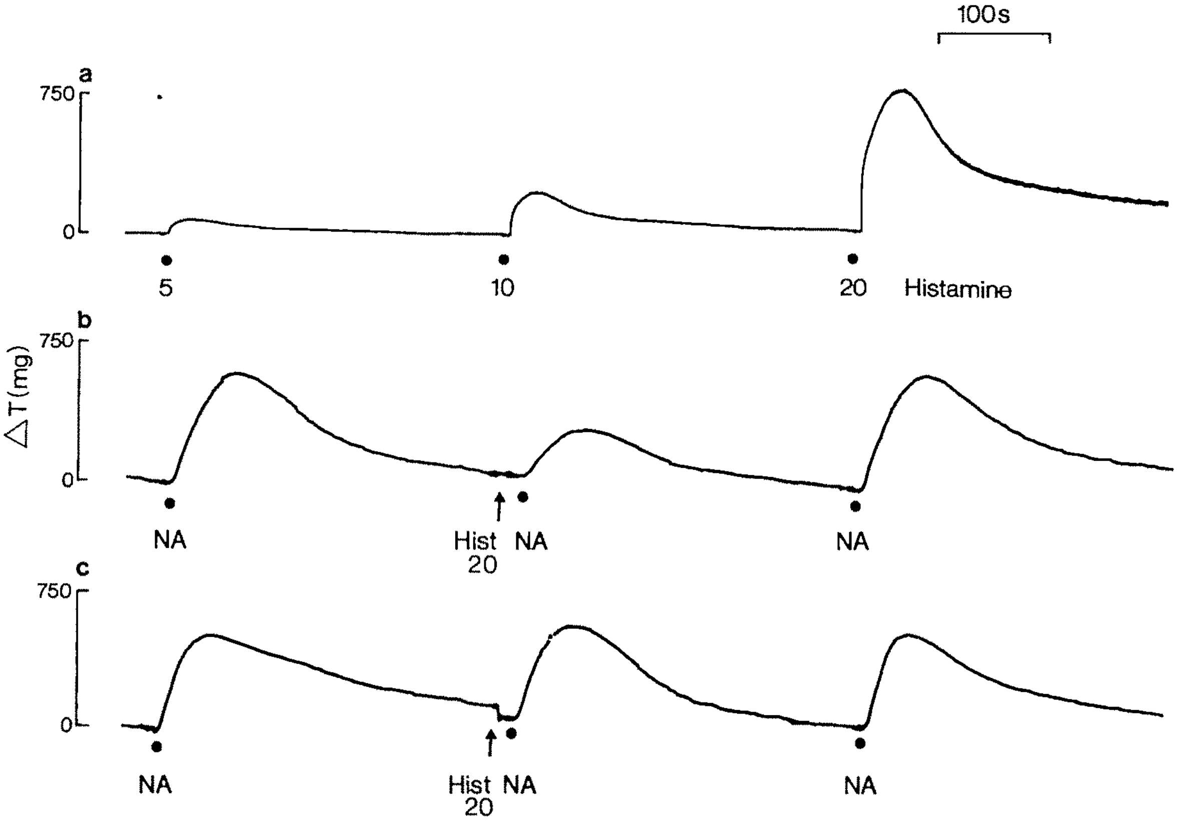

**Figure 1** Rabbit aortic strip. (a) Contractile responses to histamine (at dots) in normal Krebs solution. Doses are shown in µg. (b) In the presence of mepyramine, 1 µg/ml. Histamine (Hist), 20 µg had no contractile effect but reduced the otherwise constant contractile response to noradrenaline (NA), 0.25 µg. (c) In the presence of mepyramine, 1 µg/ml and metiamide, 1 µg/ml. Histamine (Hist), 20 µg had no effect on the response to noradrenaline (NA), 0.25 µg.

FT03 force-displacement transducer and recorded on a Grass polygraph.

In experiments in which vasoconstrictor responses to histamine were studied three or four doses which were found to cause responses on the linear part of the dose-response curve were used. Each dose was repeated 3 times and the mean response used to plot a dose-response curve; changes in sensitivity were expressed by comparing equi-active dose ratios.

Vasodilator responses were studied by the method of Carroll & Glover (1973). Mepyramine was added to the perfusate if necessary to abolish the constrictor ($H_1$) action of histamine. A dose of noradrenaline was chosen which gave a reproducible vasoconstrictor response (rise in perfusion pressure or increase in tension). Histamine was injected at a constant time (in the range 10–30 s) before such an injection, and a vasodilator response to histamine was expressed as the percentage fall in the otherwise constant vasoconstrictor response.

Spontaneously beating rabbit atria were suspended in a 20 ml organ bath containing Krebs bicarbonate solution. Contractions were measured with a Grass FT03 force-displacement transducer and recorded on a Grass polygraph. Drugs were added directly to the bathing solution.

All drugs were dissolved in 0.9% w/v NaCl solution containing ascorbic acid 50 µg/ml and all experiments were carried out at 37°C.

### *Drugs*

The following drugs were used: dithiothreitol (Calbiochem), histamine diphosphate (Fluka, A.G.), mepyramine maleate (May & Baker), metiamide (Smith, Kline & French) and noradrenaline acid tartrate (Winthrop). The amounts stated in the text refer to these compounds with the exception of noradrenaline where the amount stated refers to the base.

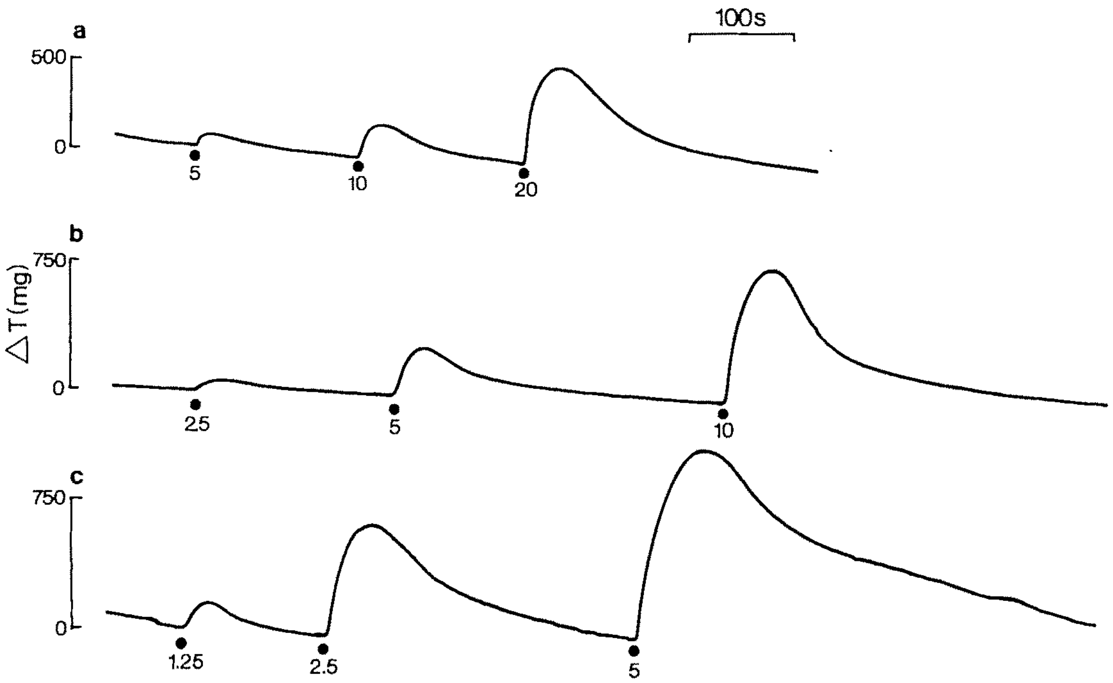

**Figure 2** Rabbit aortic strip. Contractile responses to histamine (at dots). Doses are shown in μg. (a) In normal Krebs solution; (b) in the presence of metiamide, 10 μg/ml; (c) in the presence of dithiothreitol, 154 μg/ml.

## Results

### *Histamine receptors in aortic strips*

When injected in doses of 1 to 100 μg into the perfusion stream, histamine produced brief, reproducible dose-related contractions of twelve aortic strips (Figures 1a and 2). This action of histamine was mediated by $H_1$-receptors since the contractile responses to these doses were abolished in the presence of mepyramine (10 ng to 1 μg/ml).

In the presence of mepyramine, histamine had no effect on the tension of the aortic strips. However, when injected before a dose of noradrenaline, histamine produced dose-dependent reductions in the otherwise constant contractile response. This vasodilator action of histamine was abolished by metiamide and can therefore be attributed to $H_2$-receptor activity. Figure 1b and c shows records from a typical experiment and 5 dose-response curves are illustrated in Figure 3. In eight similar experiments histamine (10 μg), in the absence of mepyramine, caused a tension increase of 603 ± 56 mg (mean ± s.e.) and, in the presence of mepyramine (1 μg/ml), a reduction of 46 ± 9% in the contractile response to noradrenaline. The dose of noradrenaline, while constant in any one experiment, was in the range 0.1 to 2.5 μg and resulted in tension changes of 630 ± 45 mg.

In the rabbit ear artery the constrictor response to histamine is potentiated by metiamide and hence represents a balance between a predominant constrictor action mediated by mepyramine-sensitive $H_1$-receptors and a vasodilator action mediated by metiamide-sensitive $H_2$-receptors (Glover *et al.*, 1973). In the present experiments, metiamide (10 μg/ml) caused a 2.7 ± 1.3 fold potentiation of the responses of five aortic strips to histamine, as calculated from the shifts to the left of the histamine dose-response curves. The effect of metiamide on the responses of one of these preparations to histamine is illustrated in Figure 2 and a typical dose-response curve is shown in Figure 4.

### *Effects of dithiothreitol on $H_1$-responses to histamine*

(a) *Aortic strip.* As shown by Fleisch *et al.* (1973), the contractile responses of rabbit aortic strips to histamine were potentiated by DTT. A typical record is shown in Figure 2. In seven experiments DTT (154 μg/ml i.e. 1 mM) potentiated the response to histamine 6.5 ± 2.1 times as indicated by the shifts to the left of the histamine dose-response curves.

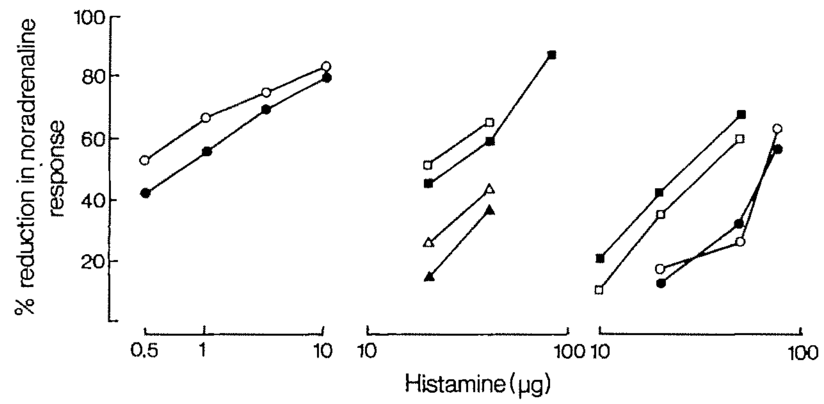

**Figure 3** Vasodilator action of histamine in rabbit aorta: effect of dithiothreitol. In the presence of mepyramine, histamine was injected before a dose of noradrenaline and the histamine response expressed as the percentage reduction in the otherwise constant contractile response to noradrenaline. Paired dose-response curves obtained in five experiments. Closed symbols—control responses, open symbols—in presence of DTT 154 μg/ml. Each point is the mean of three observations. In no case was the s.e. mean greater than 5.

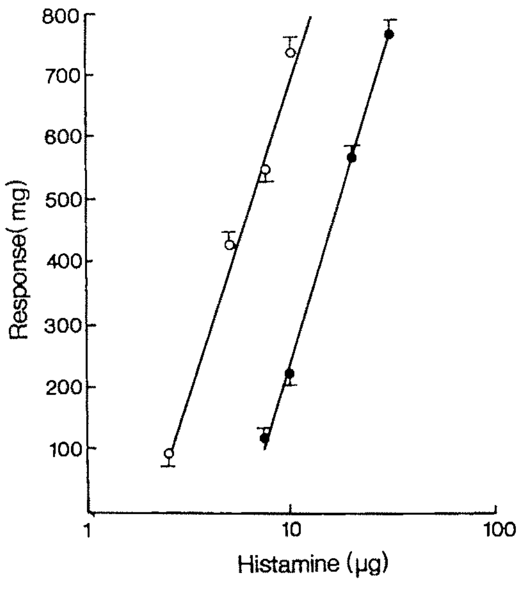

**Figure 4** Effect of metiamide on the constrictor response of the rabbit aortic strip to histamine. (●) Control, (O) metiamide 10 μg/ml. Each point is the mean of three observations and vertical bars indicate s.e. mean.

(b) *Ear arteries.* DTT (154 μg/ml) had no effect on the tension of aortic strips, but in three out of eight experiments caused small increases (5–15 mmHg) in the perfusion pressure of the rabbit ear arteries. In every case DTT caused a marked potentiation of the responses of the ear arteries to histamine. The onset of potentiation was rapid and the responses remained stable for 60–90 min although there was generally some deterioration after this time. Dose-response curves were obtained to histamine on the eight arteries before and 30 min after the addition of DTT (154 μg/ml). The addition of DTT produced a shift to the left of the histamine dose-response curves and, as indicated by these shifts, a 79.0 ± 29.1 times potentiation of the responses to histamine.

(c) *In the presence of metiamide.* Metiamide causes potentiation of histamine vasoconstrictor responses by blocking $H_2$-receptors. DTT, however, still caused marked potentiation of the constrictor responses of both the aorta and the ear artery in the presence of a maximal degree of $H_2$-receptor blockade with metiamide. In this series of experiments the concentration of metiamide was progressively increased until a point was reached where doubling the concentration produced no further potentiation of the response to histamine. The concentration of metiamide required to achieve this was 40 to 100 μg/ml. Under these conditions the responses of five ear arteries were potentiated 5.1 ± 1.7 times and the responses of two aortic strips 2.1 and 2.4 times. However, with this maximum blockade of the $H_2$-receptors the addition of DTT (154 μg/ml) potentiated the responses of the five ear arteries to histamine a further 25.1 ± 5.9 times and the responses of the two aortic strips a further 3.2 and 3.7 times. The results obtained from one of the ear arteries are shown in Figure 5.

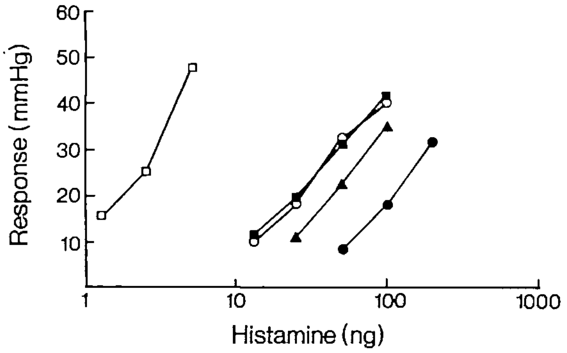

**Figure 5** Effect of metiamide and metiamide plus dithiothreitol (DTT) on the constrictor response of the rabbit ear artery to histamine. (●) Normal Krebs, (▲) metiamide 10 μg/ml, (○) metiamide 20 μg/ml, (■) metiamide 40 μg/ml and (□) metiamide 40 μg/ml plus DTT 154 μg/ml. Each point is the mean of three observations. In no case was the s.e. mean greater than 3 mmHg.

*Effects of dithiothreitol on $H_2$-responses to histamine*

(a) *Aorta and ear artery.* In the presence of mepyramine (10 ng to 1 μg/ml) the constrictor response of both vessels to histamine was abolished and a vasodilator action revealed. In each of ten ear arteries and five aortic strips histamine (1 to 50 μg) caused reproducible reductions in the responses to noradrenaline. This action of histamine was unaffected by DTT (154 μg/ml) but was abolished by metiamide (1 to 10 μg/ml). A typical record from one aortic strip is illustrated in Figure 6, and the effect of DTT on the responses of both vessels summarized in Figures 3 and 7.

Following the addition of DTT to the perfusate the dose of noradrenaline was adjusted where necessary to maintain the vasoconstrictor response at the pre-DTT level. The ratio of noradrenaline doses used therefore provided an index of any change in sensitivity to noradrenaline caused by DTT. The results are summarized in Table 1a and b.

In three ear arteries DTT had no effect on the response to noradrenaline but in 7 it was potentiated 1.5–4.0 times (mean of 10 experiments = 2.2 ± 0.37).

In contrast, the responses of all 5 aortic strips to noradrenaline were reduced by DTT, and it was necessary to increase the dose of noradrenaline in each case to maintain a constant constrictor response (mean of 5 experiments = 8.5 ± 2.7).

(b) *Human temporal artery.* Histamine (0.1 to 25 μg) had no constrictor action on this preparation and, in the absence of mepyramine, produced dose-dependent reduction in the constrictor responses to noradrenaline. In each of five experiments this action of histamine was unaffected by DTT (154 μg/ml) (Figure 8) but was abolished by metiamide (1 to 10 μg/ml).

DTT had no consistent effect on the responses of these arteries to noradrenaline. Table 1c shows that in 1 experiment the response was unchanged, in 2 it was slightly increased (2 times) and in 2 it was slightly reduced (0.6 and 0.8 times).

(c) *Rabbit atria.* In 6 spontaneously beating rabbit atria preparations histamine (2.5 μg/ml for 1 min) produced an increase in the force of contraction (mean maximum 71.8 ± 9.4%). This action of histamine was unchanged (71.6 ± 9.9%) in the presence of DTT (154 μg/ml) but was greatly reduced (11.2 ± 3.2%) by metiamide (5 μg/ml). A typical experiment is illustrated in Figure 9.

## Discussion

The results show that the rabbit aorta, like the ear artery (Glover *et al.*, 1973), contains both $H_1$ and $H_2$ histamine receptors. Thus, although histamine normally has a contractile or vasoconstrictor action on these isolated vessels, a metiamide-sensitive vasodilator action is revealed when the $H_1$-receptors are blocked with mepyramine. In order to demonstrate vasodilator responses in isolated artery preparations it is usually necessary first to raise their tone by vasoconstrictor stimulation (de la Lande & Rand, 1965; Starr & West, 1966). Alternatively, a vasodilator stimulus may be applied before a vasoconstrictor stimulus, and the vasodilator response recorded as a reduction in the otherwise constant vasoconstrictor response (Carroll & Glover, 1973). This was the method used to demonstrate $H_2$-receptor activity in rabbit ear and human temporal arteries (Glover *et al.*, 1973), and we have now applied it to rabbit aortic strips. Histamine normally contracted these strips; however, after mepyramine it had no effect on the resting tension but caused a dose-related reduction in the response to noradrenaline. This action of histamine was abolished by metiamide and hence can be attributed to stimulation of $H_2$-receptors. In addition, in both the rabbit ear artery and aorta the normal response to histamine is a balance between a predominant constrictor action mediated by $H_1$-receptors and a vasodilator action mediated by $H_2$-receptors, since it is abolished by mepyramine and potentiated by metiamide.

Our results confirm the finding that DTT potentiates the contractile response of the rabbit aorta to histamine (Fleisch *et al.*, 1973; Fleisch, Krzan & Titus, 1974), and we have extended this observation to the rabbit ear artery. In our aortic strip experiments, 3–4 graded doses of histamine were injected into the

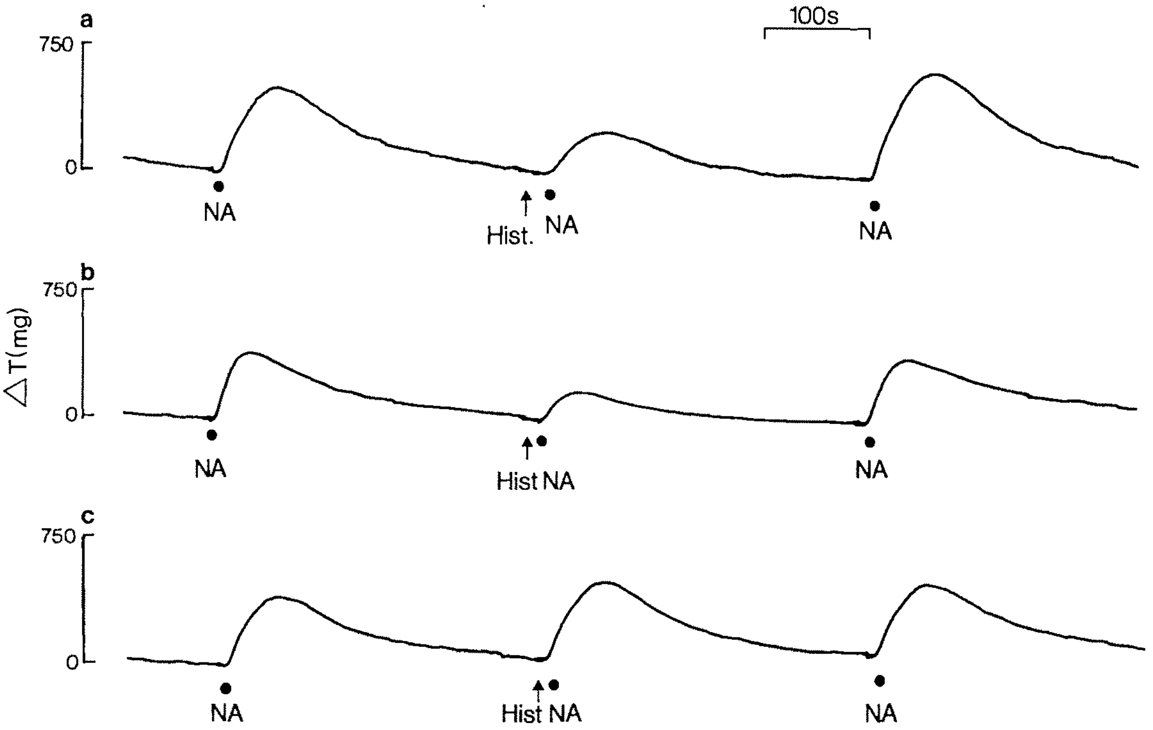

**Figure 6** Rabbit aortic strip. All records were obtained in the presence of mepyramine, 1 μg/ml. (a) Histamine (Hist), 25 μg had no contractile effect but reduced the contractile response to noradrenaline (NA), 0.1 μg. (b) In the presence of dithiothreitol (DTT), 154 μg/ml this effect of histamine was unchanged. (c) In the presence of metiamide, 1 μg/ml this effect of histamine was abolished. Note that in the presence of DTT and metiamide it was necessary to increase the dose of noradrenaline (NA) to 0.5 μg to keep the contractile response constant.

Krebs solution which flowed rapidly through the organ bath, in contrast to the cumulative additions used by Fleisch *et al.* (1973; 1974). The responses were therefore brief, thus allowing 3 points on the straight part of the dose-response curve to be obtained rapidly. We did not use doses which would elicit a maximal response, but the method has the advantage that the desensitization which follows cumulative additions and a maximal response to histamine does not occur, and the responses were reproducible. In the absence of a maximal response to the $ED_{50}$ (concentration of agonist required to elicit 50% of the maximal response) cannot be calculated, but DTT caused a shift of the dose-response curve to the left with a dose-ratio of 6.5 ± 2.1. This compares well with the $ED_{50}$ ratios reported by Fleisch *et al.* in 1973 and 1974 of 10 and just over 7 respectively. DTT caused an even greater potentiation of the responses of the ear artery to histamine. It is not possible to obtain maximum responses and full dose-response curves using the perfused artery method, but in these preparations we found that DTT caused a marked shift to the left of the dose-response curve with a dose-ratio of 79 ± 29.

Fleisch *et al.* (1973) concluded that the enhancement of the constrictor response of the rabbit aorta to histamine produced by DTT was due to an increase in the activity of the histamine receptor system. Subsequently (1974) they found that, in contrast, DTT depressed the response of the guinea-pig aorta to histamine, and suggested that this could be explained by the existence of different histamine $H_1$-receptor sub-types. Our finding that the rabbit aorta and ear artery contain both $H_1$ and $H_2$ histamine receptors, and that the contractile response to histamine was increased when the $H_2$-receptors were blocked with metiamide raised another possibility, that is, that DTT caused potentiations not by increasing the activity of $H_1$-receptors, but by decreasing the activity of $H_2$-receptors.

However, our results show that DTT still caused marked potentiations of the constrictor response to histamine in the presence of the maximal degree of $H_2$-receptor blockade produced by metiamide. It seems reasonable to conclude that DTT does cause an increase in the activity of $H_1$-receptor systems in these rabbit arteries. This finding does not exclude the possibility of a concurrent action, either blockade or

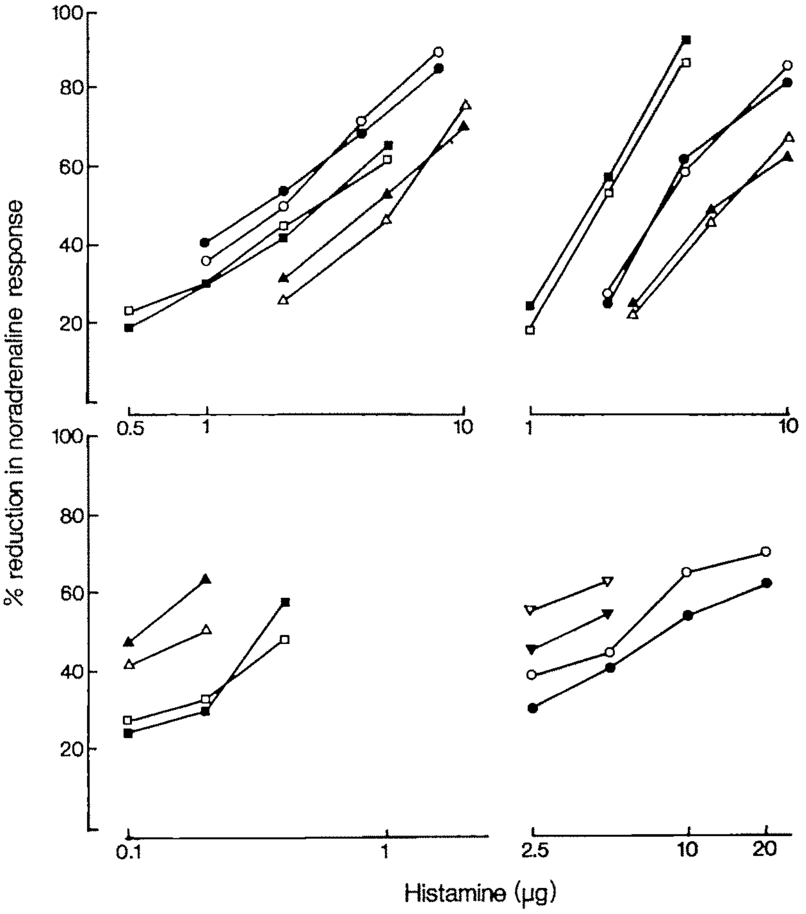

**Figure 7** Vasodilator action of histamine in rabbit ear artery: effect of dithiothreitol (DTT). Responses expressed as in Figure 3. Paired dose-response curves obtained in ten experiments. Closed symbols—control; open symbols—DTT 154 µg/ml. Each point is the mean of three observations. In no case was the s.e. mean greater than 5.

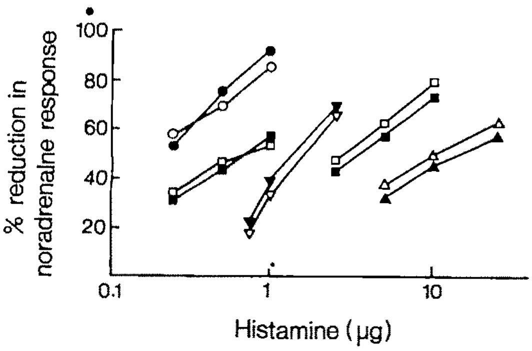

**Figure 8** Vasodilator action of histamine in human temporal artery: effect of dithiothreitol (DTT). Responses expressed as in Figure 3. Paired dose-response curves obtained in five experiments. Closed symbols—control; open symbols—DTT 154 µg/ml. Each point is the mean of three observations. In no case was the s.e. mean greater than 5.

potentiation of $H_2$-receptor systems. However, after $H_1$-receptor blockade with mepyramine, the $H_2$-receptor effect, revealed as a depression of the constrictor response to noradrenaline, was unaffected by DTT. It is unlikely that mepyramine was modifying a possible effect of DTT on the $H_2$-receptors in these vessels, unless there are sub-types of $H_2$-receptors, since the responses of the human temporal artery were unaffected by DTT. In the temporal artery, histamine has no vasoconstrictor action and it was not necessary to use mepyramine to reveal the $H_2$-response. Further, the positive inotropic action of histamine in the isolated rabbit atria was unaffected by DTT. It can therefore be concluded that these cardiovascular responses mediated by $H_2$-receptors are unaffected by DTT.

Fleisch *et al.* (1973) showed that the potentiating action of DTT was specific for histamine contractile responses in the rabbit aorta since DTT caused a

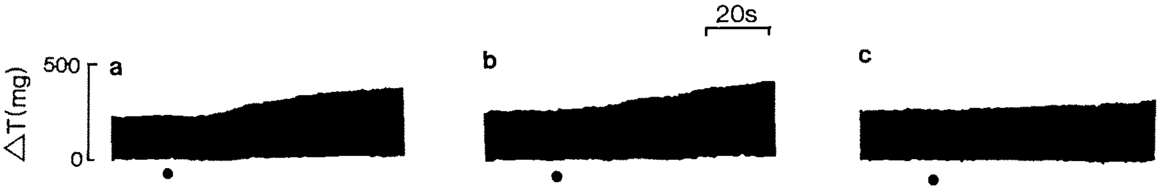

**Figure 9** Spontaneously beating rabbit atria. Responses to histamine (at dots), 2.5 μg/ml. (a) In normal Krebs solution; (b) in the presence of dithiothreitol 154 μg/ml; (c) in the presence of metiamide 5 μg/ml.

**Table 1** Change in sensitivity to noradrenaline caused by dithiothreitol (DTT) in ear artery and aorta of the rabbit and in human temporal artery

(a) *Rabbit ear artery*

| *Control* | | *DTT* | | *Ratio control/DTT* | |
|---|---|---|---|---|---|
| *Dose (ng)* | *Response (mmHg)* | *Dose (ng)* | *Response (mmHg)* | *Dose* | *Response* |
| 2 | 25 | 2 | 23 | 1.0 | 1.09 |
| 4 | 24 | 4 | 20 | 1.0 | 1.2 |
| 5 | 19 | 5 | 21 | 1.0 | 0.9 |
| 7.5 | 30 | 5 | 28 | 1.5 | 1.07 |
| 4 | 27 | 2 | 31 | 2.0 | 0.87 |
| 20 | 24 | 10 | 27 | 2.0 | 0.89 |
| 2.5 | 29 | 1 | 31 | 2.5 | 0.94 |
| 3 | 26 | 1 | 25 | 3.0 | 1.08 |
| 4 | 33 | 1 | 35 | 4.0 | 0.94 |
| 20 | 30 | 5 | 33 | 4.0 | 0.91 |
| | | | Mean ± s.e. | 2.2 ± 0.37 | 0.99 ± 0.04 |

(b) *Rabbit aorta*

| *Control* | | *DTT* | | *Ratio control/DTT* | |
|---|---|---|---|---|---|
| *Dose (μg)* | *Response (mg)* | *Dose (μg)* | *Response (mg)* | *Dose* | *Response* |
| 0.3 | 690 | 1 | 665 | 0.3 | 1.04 |
| 0.25 | 630 | 1 | 595 | 0.25 | 1.06 |
| 0.1 | 510 | 0.5 | 410 | 0.2 | 1.24 |
| 0.1 | 440 | 1.5 | 420 | 0.07 | 1.05 |
| 0.1 | 905 | 1.5 | 780 | 0.07 | 1.16 |
| | | | Mean ± s.e. | 0.18 ± 0.05 | 1.11 ± 0.04 |

(c) *Human temporal artery*

| *Control* | | *DTT* | | *Ratio control/DTT* | |
|---|---|---|---|---|---|
| *Dose (ng)* | *Response (mmHg)* | *Dose (ng)* | *Response (mmHg)* | *Dose* | *Response* |
| 100 | 24 | 50 | 22 | 2.0 | 1.09 |
| 250 | 22 | 400 | 25 | 0.6 | 0.88 |
| 25 | 23 | 30 | 24 | 0.8 | 0.96 |
| 50 | 29 | 50 | 27 | 1.0 | 1.07 |
| 500 | 28 | 250 | 32 | 2.0 | 0.88 |
| | | | Mean ± s.e. | 1.28 ± 0.30 | 0.98 ± 0.05 |

slight reduction in the maximal response to KCl, increased the values of $ED_{50}$ for noradrenaline and 5-hydroxytryptamine approximately 4.5 fold and completely abolished the response to angiotensin. We have also found that DTT caused slight depression of the contractile response to noradrenaline in the rabbit aorta. However, we have not studied the effect of DTT on the dose-response curves of noradrenaline and other vasoconstrictor drugs in the rabbit ear artery, but our results show that whereas DTT caused a 79 ± 29 fold potentiation of the constrictor response to histamine, it caused only slight potentiation (2.2 ± 0.37) of the response to noradrenaline. It seems reasonable to conclude that the marked potentiation of the histamine constrictor response caused by DTT is not due to a non-specific action on the vascular smooth muscle. Whether or not there are responses mediated by receptors other than adrenoceptors which are potentiated by DTT, we can conclude that DTT modifies $H_1$ but not $H_2$-receptor systems in the rabbit aorta and ear artery, and to this extent is highly specific.

Enhancement of the histamine response in the rabbit aorta is not due to inhibition of histamine-metabolizing enzymes, since histaminase activity is negligible in this tissue and histamine-*N*-methyltransferase activity was inhibited only 10% by DTT (Fleisch *et al.,* 1973). Our finding that the $H_2$-responses are not potentiated by DTT supports this conclusion and in addition makes it unlikely that the potentiation could be due to an inhibition of histamine uptake in these tissues.

It is well known that DTT reduces disulphide linkages to sulphydryl groups, and Fleisch *et al.* (1973) concluded that disulphide bridges must occupy prominent positions either in histamine receptors or along the subsequent excitation-contraction pathway. Our results show that this conclusion can be applied to certain cardiovascular $H_1$ but not $H_2$ histamine receptor responses in the rabbit.

This work was supported by a grant from the National Health and Medical Research Council of Australia. We are grateful to Dr W.A.M. Duncan of Smith, Kline & French, England for kindly supplying the metiamide used in this study.

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*(Received July 9, 1976.*
*Revised September 28, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 343–347

# THE EFFECTS OF BRADYKININ AND PROSTAGLANDIN $E_1$ ON RAT CUTANEOUS AFFERENT NERVE ACTIVITY

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1 The activity produced by intra-arterial bradykinin and prostaglandin $E_1$ was investigated in multifibre strands dissected from the saphenous nerves of anaesthetized rats.

2 Bradykinin (0.5–10 μg) alone produced little activity in nerve strands but produced considerable activity following a 10 min infusion, but not a single injection, of prostaglandin $E_1$ (5–100 ng).

3 Prostaglandin $E_1$ alone produced a few large height spikes but following several injections of bradykinin smaller height spikes were also produced by prostaglandin $E_1$.

4 It was concluded that the presence of a low concentration of prostaglandin $E_1$ is required for bradykinin to manifest its action and that bradykinin and prostaglandin $E_1$ are mutually potentiating in their effects on afferent nerve terminals.

## Introduction

Prostaglandin generation has now been shown to take place in many forms of damage to the skin (Greaves & Søndergaard, 1970; Änggård & Jonsson, 1971). It was suggested by Ferreira (1972) that the prostaglandins sensitize 'pain' receptors to stimulation by other inflammatory mediators, and it has been widely accepted that inhibition of synthesis of prostaglandins constitutes the principal mechanism of action of the non-steroidal anti-inflammatory agents. This paper reports the electrophysiological effects of bradykinin and prostaglandin $E_1$ both alone and in combination on cutaneous afferent nerve strands of the rat.

## Methods

The method used by Fjällbrant & Iggo (1961) was adapted for use in rats. Male albino rats, 220–270 g, were anaesthetized with urethane (1250–1500 mg/kg i.p.). The trachea and jugular vein were cannulated and an incision was made in the skin of the right hind limb overlying the saphenous nerve. A thread was placed under the femoral artery proximal to branching of the saphenous artery and branches other than the saphenous artery were ligated. A cannula was then inserted in a retrograde direction into the femoral artery distal to branching of the saphenous artery. During insertion of the cannula slight tension was exerted on the thread under the femoral artery to prevent blood loss. The cannula was filled with Tyrode solution before insertion and connected to a three-way tap. Provided there was no leak in the cannula or tap there was no backflow of blood into the cannula and hence heparin was not used in these experiments. The dorsal surface of the right hind limb was then partially embedded in gypsum and a liquid paraffin pool was made over the saphenous nerve. Recordings were made from dissected multifibre strands of the nerve using silver wire electrodes. The signals were amplified and simultaneously displayed on an oscilloscope and recorded on magnetic tape. Drugs in volumes 0.05–0.1 ml were usually injected into the arterial cannula over a period of 30 s and followed by 0.1 ml of Tyrode solution injected over the following 30 seconds. A control injection of 0.2 ml of Tyrode solution was tested at the start of each experiment. Activity in the strands was observed for a minimum time of 10 min following an injection. In some experiments prostaglandin $E_1$ solution (0.5–1.0 ml) was given as a slow infusion over 10 minutes. In these experiments the response to infusion of a similar volume of Tyrode solution was also tested. The response to mechanical stimulation by stroking the skin was used as an indication of the stability of the recording conditions and the functional state of the strand.

Since it was suggested by Crunkhorn & Willis (1971) that prostaglandins of the E-type produce inflammatory effects by releasing amines from mast cells, some experiments were performed in rats pretreated with compound 48/80 to deplete mast cells of histamine and 5-hydroxytryptamine. Rats were

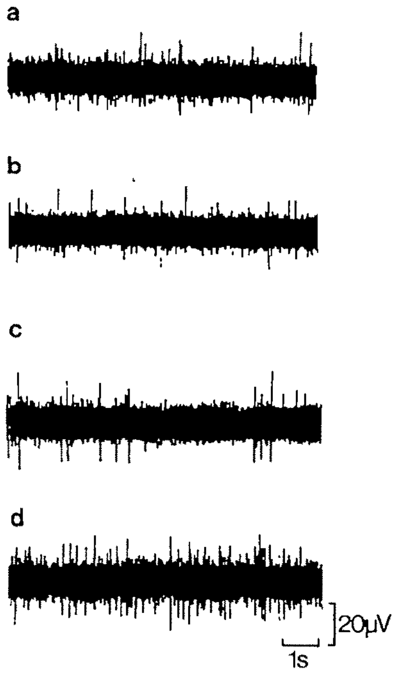

**Figure 1** Effect of 10 min infusion of prostaglandin $E_1$ (5 ng) on response to bradykinin (0.5 μg). Control responses were recorded in the minute preceding the bradykinin injections. (a) Control; (b) 1 min after injection of bradykinin; (c) 20 min later—control after infusion of prostaglandin $E_1$; (d) 1 min after 2nd injection of bradykinin (note large spikes).

pretreated with twice daily injections of compound 48/80 intraperitoneally for 4–10 days before the experiment. For the first six injections a dose of 0.6 mg/kg was used and thereafter the dose was increased to 1.2 mg/kg. The strain of rats used appeared to be more difficult to deplete than those used by Di Rosa, Giroud & Willoughby (1971) who used a similar regime, since even after several days treatment with compound 48/80 the feet were often swollen on the day of the experiment. Therefore 4 rats were given two intra-arterial injections of compound 48/80 (1 rat–0.1 ml of 10 μg/ml; 3 rats–0.1 ml of 100 μg/ml) at the start of the experiment.

### *Drugs*

The following were used: bradykinin triacetate (Sigma), compound 48/80 (Wellcome), 5-hydroxytryptamine creatinine sulphate (Sigma) and prostaglandin $E_1$ (Upjohn), concentrations and doses being expressed as such.

## Results

### *Bradykinin*

Bradykinin was injected intra-arterially at the start of 18 experiments before injection of any other drug, in doses varying from 0.5 μg to 10 μg. In 13 of these experiments very little change in activity in the nerve strands was recorded and in the other 5 experiments no change in activity was apparent (e.g. Figure 1). In only 1 experiment was there marked response to bradykinin (10 μg).

### *Prostaglandin $E_1$*

Injection of prostaglandin $E_1$ in doses 5–100 ng initiated low frequency, irregular firing in a very few fibres in strands from 12 rats which had been given no other drugs, but in 3 rats there was no response. The response was slow in onset, usually occurring 2–5 min after the injection started. The response, although slight, was often long-lasting and sometimes present for as long as 20 minutes. The spikes were large in height and in 3 experiments the response was compared with the response of the same strand to 5-hydroxytryptamine (1–3 μg). It was found that more units were excited by 5-hydroxytryptamine than by prostaglandin $E_1$ and that more smaller height spikes were produced. The onset of action of 5-hydroxytryptamine was also more rapid than that of prostaglandin $E_1$, usually starting within the 30 s injection time of the 5-hydroxytryptamine dose. In 5 experiments, where higher doses of prostaglandin $E_1$ (1–10 μg) were tested, no greater response was obtained than with lower doses.

The response to prostaglandin $E_1$ was still present in 4 out of 5 rats which had been pretreated with twice daily intraperitoneal injections of compound 48/80, 4–10 days before the experiment, and also in 3 out of 4 rats pretreated with two intra-arterial injections of compound 48/80 at the start of the experiment.

### *Prostaglandin $E_1$ after bradykinin*

Prostaglandin $E_1$ injections (6 experiments) or infusions (15 experiments) 5–100 ng, were given following an initial injection of bradykinin (0.5–10 μg). In these experiments the response to prostaglandin $E_1$ did not obviously differ from the response in those experiments where it was infused before any other drug. However is experiments where 2 further injections of bradykinin were given it was noted that there was potentiation of the response to injection of 10 ng prostaglandin $E_1$ (Figure 2).

### *Bradykinin after prostaglandin $E_1$*

In 6 experiments the response to injection of bradykinin, 0.5–10 μg, was not potentiated following a

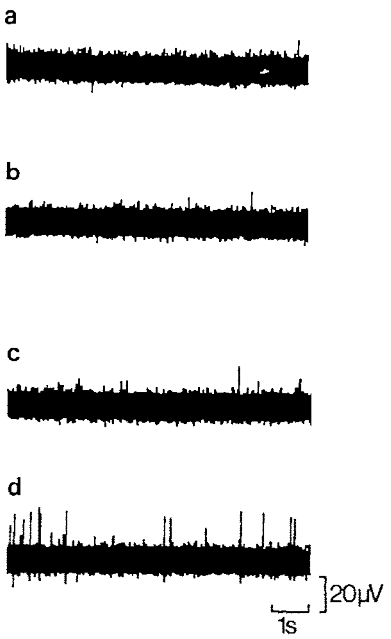

**Figure 2** Potentiation of response to injection of prostaglandin $E_1$ (10 ng) by several injections of bradykinin (10 µg). Control responses were recorded during the minute preceding the prostaglandin injections. (a) Control 40 min after 1st injection of bradykinin; (b) 1 min after injection of prostaglandin $E_1$; (c) 80 min later—control 20 min after 2 further injections of bradykinin; (d) 1 min after injection of prostaglandin $E_1$ (note greater number of large and small spikes).

single injection of prostaglandin $E_1$ or by simultaneous injection of prostaglandin $E_1$ (5–100 ng). However, marked potentiation of the bradykinin response following a 10 min infusion of prostaglandin $E_1$ (5–100 ng) occurred in 9 out of 13 experiments (e.g. Figure 1). A 10 min infusion of the same volume of Tyrode solution did not increase the response to bradykinin. The bradykinin response after infusion of prostaglandin $E_1$ started within 30 s of completion of the injection i.e. during the Tyrode injection. Potentiation of either or both small height spike responses and/or large height spike responses were seen. These differences were probably due to differences in the size of strands as well as in the units contained in the strands. In those experiments where there was no potentiation it was found that the response to mechanical stimulation by stroking the skin was reduced, indicating that recording conditions had altered, probably due to leakage of fluid around the strand. This was a major problem in these experiments because of oedema production by the drugs. The greatest potentiations of bradykinin were found after infusion of 5–10 ng of prostaglandin $E_1$.

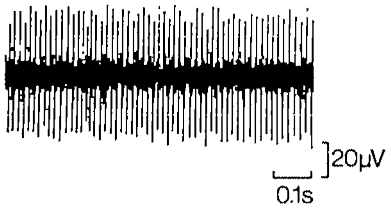

**Figure 3** Rapid firing of a unit 1 min after injection of bradykinin (5 µg) preceded by a 10 min infusion of prostaglandin $E_1$ (5 ng).

The duration of the potentiation was difficult to assess since there was a gradual increase in activity in the strands during the course of the experiments. However it appeared to be relatively short-lived since in 3 experiments where a second injection of bradykinin was given, it was not as effective as the first following the infusion of prostaglandin $E_1$.

In 2 experiments bursts of activity of a unit with a regular firing rate occurred following the injection of bradykinin after infusion of prostaglandin $E_1$ (Figure 3). In one experiment the rate of firing of the unit reached a maximum of 170/second. The longest burst lasted for 90 seconds. In this experiment the unit was then found to be responsive to touching a region of the skin stretched to form the paraffin pool. A second infusion of bradykinin 1 hour later (following a second infusion of prostaglandin $E_1$) also produced firing of this unit but the firing rate was less and the duration was shorter.

## Discussion

These experiments show that the action of bradykinin is potentiated by infusion but not by single injection of prostaglandin $E_1$. Ferreira (1972) using intradermal injections into human skin, and Juan & Lembeck (1974) using the reflex fall in blood pressure of rabbits as an index of the nociceptive action of bradykinin, also found that infusions of prostaglandin $E_1$ were more effective than single injections in potentiating bradykinin. Prostaglandin $E_1$ appeared to potentiate the production of small and large height spikes by bradykinin and in 2 experiments marked repetitive firing of a unit, with the characteristics of a slowly adapting mechanoreceptor type II, occurred. One of these units had its receptive field in a region of the skin under stretch to form the paraffin pool and it would seem that bradykinin lowered the threshold of the receptor to stretch.

Bradykinin alone had little action in most of the rat preparations and large doses were necessary to produce any effect. Fjällbrant & Iggo (1961) found that impure bradykinin had little action on C fibres from the cat saphenous nerve but other workers have shown that doses of bradykinin in the microgram range induced a response in afferent nerves (Beck & Handwerker, 1974; Mense & Schmidt, 1974; Franz & Mense, 1975). The question thus arises as to whether bradykinin is an effective algesic agent in its own right or whether it requires the presence of a low concentration of a prostaglandin or a prostaglandin precursor for its action to be manifest. Marked potentiation of bradykinin (0.5–10 μg) occurred with 5–100 ng of prostaglandin $E_1$ infused over 10 min but other doses of either bradykinin or prostaglandin $E_1$ were not used in these experiments. It is possible that much lower doses of prostaglandin $E_1$ might have been effective in potentiating bradykinin, and that the marked responses to bradykinin of afferent units described by other workers (Beck & Handwerker, 1974; Mense & Schmidt, 1974; Franz & Mense, 1975), and also in one experiment reported here, might have been due to the presence of low levels of prostaglandins released under the conditions of the experiments, including perhaps the use of heparin which in high doses has been shown to release prostaglandins (Damas & Deby, 1974). The potentiating effect of prostaglandin $E_1$ is probably dose-related since Moncada, Ferreira & Vane (1975) showed in the dog knee joint that bradykinin became more effective in inducing pain as the prostaglandin level increased in the joint cavity. It is significant also that the two observations which established bradykinin as a potent algesic agent were (a) its pain-producing action on the cantharidine blister base (Keele & Armstrong, 1964), an inflamed site probably having high local levels of prostaglandins, and (b) its pain-producing action when injected into the spleen of dogs (Guzman, Braun & Lim, 1962) where a basal resting release of E-type prostaglandins has since been shown to occur (Ferreira, Moncada & Vane, 1973). Nevertheless the possibility remains that the action of bradykinin on small fibres might have been less obvious in the multifibre strands used in the present experiments than on the single units used by other workers (Beck & Handwerker, 1974; Mense & Schmidt, 1974; Franz & Mense, 1975). It is also possible that there might be a species difference between cats (used by the previous workers) and rats (used in the present experiments) in the ability of bradykinin itself to release prostaglandins, an action which bradykinin has been shown to have at some sites e.g. in dog spleen (Ferreira *et al.*, 1973).

The injection or infusion of prostaglandin $E_1$ produced firing of a few spikes which appeared from their height to be from A fibres. This response differed from that to bradykinin in the presence of prostaglandin $E_1$, which usually produced many small height spikes and some large spikes, and also from that to 5-hydroxytryptamine which produced many small and some large height spikes. It is possible that some of the larger height spikes were from mechanoreceptors activated by increased interstitial fluid pressure resulting from fluid leakage from the circulation during the course of the experiments. Such a response would be relatively slow in onset and could account for the long latency response to prostaglandin $E_1$. The response to prostaglandin $E_1$ did not appear to be due to release of histamine or 5-hydroxytryptamine from mast cells since it was not abolished by pretreatment with compound 48/80.

Although the experiments were not primarily designed to show potentiation of prostaglandin $E_1$ by bradykinin, it was found that after several injections of bradykinin, prostaglandin $E_1$ appeared to have a greater effect; obvious firing of both small and large height spikes occurred. In view of the small quantity of prostaglandin $E_1$ (10 ng) that produced this effect, the possibility must not be discounted that prostaglandin $E_1$ might be a cause of pain in inflammatory states where bradykinin is also present over a long period.

It is concluded from the results presented here that bradykinin and prostaglandin $E_1$ are mutually potentiating in their effects on afferent nerve terminals.

Experiments on rats treated with prostaglandin synthetase inhibitors would be of value in determining whether the potentiating action of bradykinin or exogenously applied prostaglandins is due to a release by bradykinin of endogenous prostaglandins or their precursors.

We wish to thank Dr J.E. Pike of the Upjohn Company, Kalamazoo, Michigan for generous samples of prostaglandin $E_1$. Financial support for part of this work was provided by the Agricultural Research Council.

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*(Received July 19, 1976.
Revised September 24, 1976.)*

*Br. J. Pharmac.* (1977), 59, 349–351

# NATURE OF HISTAMINE RECEPTORS CONCERNED IN CAPILLARY PERMEABILITY

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1 Histamine and 2-methyl-histamine ($H_1$-receptor agonist) caused dose-dependent increases in capillary permeability in albino mice, but 4-methyl-histamine ($H_2$-receptor agonist) caused no significant increase.

2 Mepyramine ($H_1$-receptor antagonist) blocked the histamine-induced increase in capillary permeability whereas burimamide ($H_2$-receptor antagonist) produced no significant blockade of the histamine-response.

3 Combined mepyramine and burimamide pretreatment did not give any significantly greater protection than mepyramine alone.

4 The results indicate involvement of the $H_1$-receptors in histamine-induced increase in capillary permeability.

## Introduction

Increase in capillary permeability observed during the acute inflammatory process has been ascribed to the liberation of chemical mediators. The role of histamine in increasing capillary permeability was first described by Eppinger (1913) and was subsequently confirmed by Sollmann & Pilcher (1917) and Dale & Richards (1918). Lewis's (1927) classical 'triple response' induced by pricking histamine into human skin has been shown to be similar to the response evoked by thermal, chemical, mechanical and electrical injury (Dekanski, 1947; Sevitt, 1957). Histamine has also been reported by Gaddum (1948) to produce leakage of circulating proteins and protein-bound dye into the tissues in many species including man. Since then, several investigators have confirmed the role of histamine in the vasodilatation and increase in capillary permeability effects characteristic of acute inflammation (Wilhelm, 1962; Lichtenstein, Plaut, Henney & Gillespie, 1973; Maling, Webster, Williams, Saul & Anderson, 1974). However, the nature of the receptors involved in the capillary permeability effects of histamine are not known. With the delineation of $H_1$- and $H_2$-receptors for the action of histamine, it is now possible to determine the nature of the receptor type involved in the effect of histamine on capillary permeability.

The present investigation was undertaken to study the effects of $H_1$- and $H_2$-receptor agonists and antagonists on capillary permeability by the dye diffusion technique.

## Method

The present study was carried out in albino mice using the method of Whittle (1964) as modified by Macaraeg, Bianchine & Lazagna (1968). Healthy albino mice of either sex, weighing 20–25 g, were divided into groups of six animals. One group served as control and was injected with dye alone and other groups were injected with the dye, followed 5 min later by graded doses of histamine. Each animal was injected with 0.1 ml of 2% Evan's blue in the lateral tail vein and histamine was given intraperitoneally in graded doses of 0.75, 1.25, 2.5 and 5 µg as base. After half an hour of dye injection, the mice were killed by cervical dislocation and viscera were irrigated with 4–6 ml of distilled water over a clean petri-dish.

The irrigated fluid was filtered through glass wool and made up to 10 ml with distilled water and 0.1 ml of 0.1 N NaOH was added to clear the turbidity. The optical density of the fluid was determined at 590 nm with a spectrophotometer. The amount of dye which diffused out was calculated as µg/mouse and expressed as the increase times that in control animals. Similar studies were done with 2-methyl histamine ($H_1$-receptor agonist) and 4-methyl-histamine ($H_2$-receptor agonist).

The $PD_{50}$ for mepyramine (the dose causing 50% reduction in the amount of dye diffusion produced by 2.5 µg histamine/mouse) was calculated and the effect of this dose ($PD_{50}$) was seen on dye diffusion induced by graded doses of histamine and 2-methyl histamine. Similarly, the effect of burimamide (in a dose equal to the $PD_{50}$ dose of mepyramine) alone and together with mepyramine was studied on histamine-induced dye diffusion. The antagonists were given orally, 30 min before the test.

Drugs employed were histamine dihydrochloride (Ward Blenkinsop Co.), mepyramine maleate (May and Baker, Bombay), burimamide, 2-methyl histamine hydrochloride and 4-methyl histamine (SKF). The doses of histamine and 2-methyl histamine refer to the base and those of other drugs to their salts.

## Results

### *Effects of histamine, 2-methyl histamine and 4-methyl histamine on capillary permeability*

Intraperitoneal administration of graded doses of histamine (0.75, 1.25, 2.5 and 5 µg/mouse) caused dose-dependent increases in dye-diffusion into the peritoneal cavity (Table 1). 2-Methyl histamine in graded doses (0.75–5 µg/mouse) also increased permeability but it was less potent than histamine (Table 2). On the other hand, graded doses of 4-methyl histamine (0.75–15 µg/mouse) caused very little increase in dye diffusion.

### *Role of histamine receptor antagonists on histamine induced dye diffusion*

The $PD_{50}$ of the $H_1$-receptor antagonist, mepyramine, was calculated from the dose-response curve as 13.18 mg/kg. This dose also significantly ($P<0.01$) decreased the amount of dye which diffused into the peritoneal cavity after other doses of histamine although the effect decreased with high doses of histamine (Table 1). In contrast, the $H_2$-receptor antagonist, burimamide, at the same concentration (13.18 mg/kg) afforded no significant protection against dye diffusion induced by the standard graded doses of histamine. Combined mepyramine and burimamide pretreatment produced greater protection than mepyramine alone but the difference was not significant.

### *Effect of mepyramine on 2-methyl histamine-induced dye diffusion*

Mepyramine (13.18 mg/kg) pretreatment greatly reduced the amount of dye which diffused into the peritoneal cavity after the standard graded doses of 2-methyl histamine (Table 2). Results were highly significant ($P<0.01$).

**Table 1** Effect of $H_1$- and $H_2$-receptor antagonists on dye diffusion induced by histamine

| | *Dye diffusion values (× control) ± s.e.* | | | |
|---|---|---|---|---|
| *Histamine (µg)* | *No pretreatment* | *After mepyramine (M)* | *After burimamide (B)* | *After M and B* |
| 0.75 | 1.7 ± 0.13 | 1.1 ± 0.03* | 1.6 ± 0.06 | 1.0 ± 0.04*† |
| 1.25 | 2.8 ± 0.34 | 1.6 ± 0.03* | 2.6 ± 0.12 | 1.5 ± 0.14*† |
| 2.50 | 4.8 ± 0.11 | 2.9 ± 0.06* | 4.6 ± 0.18 | 2.5 ± 0.36*† |
| 5.00 | 8.9 ± 0.22 | 7.5 ± 0.57* | 8.7 ± 0.17 | 6.9 ± 0.48*† |

Mepyramine and burimamide were given orally in doses of 13.2 mg/kg 30 min before histamine.
* Significant difference compared with histamine alone ($P<0.01$); † Difference not significant when compared with mepyramine pretreatment ($P>0.05$).

**Table 2** Effect of mepyramine on dye diffusion induced by 2-methyl histamine

| | *Dye diffusion values (× control) ± s.e.* | |
|---|---|---|
| *2-Methyl histamine (µg)* | *No pretreatment* | *After mepyramine* |
| 0.75 | 1.6 ± 0.28 | 1.1 ± 0.10* |
| 1.25 | 2.2 ± 0.10 | 1.3 ± 0.07* |
| 2.50 | 3.0 ± 0.12 | 1.9 ± 0.13* |
| 5.00 | 4.4 ± 0.22 | 3.2 ± 0.11* |

Mepyramine was given orally in doses of 13.2 mg/kg 30 min before 2-methyl histamine.
* Significant difference compared with 2-methyl histamine alone ($P<0.01$).

## Discussion

Although it has been proposed that histamine receptors in blood vessels may fully account for the capillary permeability induced by intraperitoneal administration of histamine (Dale & Richards, 1918; Lewis, 1927), the nature of these receptors is not known. A duality of cardiovascular histamine receptors was suggested by the work of Folkow, Haeger & Kahlson (1948). These investigators showed that the histamine-induced decrease of blood pressure in cats was only partially attenuated by large doses of mepyramine. Later on, with the discovery of specific $H_2$-antagonists, Black, Duncan, Durant, Ganellin & Parsons (1972) showed that the depressor effect of histamine can be blocked completely by a combination of mepyramine and burimamide. Similarly, Parsons & Owen (1973) demonstrated the role of both $H_1$- and $H_2$-receptors in the cardiovascular system of dog, cat and rabbit.

In the present work we have studied the nature of the receptors involved in the histamine-induced increase in capillary permeability of peritoneal blood vessels in albino mice. When histamine, and 2-methyl histamine ($H_1$-agonist) were injected (i.p.) in graded doses they produced increases in dye diffusion while 4-methyl histamine ($H_2$-agonist) had little effect. Therefore, the unavoidable inference is that $H_2$-receptors are not concerned in the increase of capillary permeability.

In another set of experiments, the effect of $H_1$- and $H_2$-receptor antagonists was studied on the capillary permeability induced by histamine and 2-methyl histamine. It is evident from Tables 1 and 2 that pretreatment with mepyramine afforded highly significant protection against increases in the capillary permeability induced by histamine and 2-methyl histamine. On the other hand, burimamide pretreatment (in doses equivalent to mepyramine) afforded no significant protection. Moreover, burimamide combined with mepyramine pretreatment also did not show any significantly greater protection than mepyramine alone against histamine-induced increase in capillary permeability. These experiments give further support to the suggestion that in the histamine-induced increase in capillary permeability, $H_1$-receptors play a major role and $H_2$-receptors have an insignificant role.

The authors are grateful to SKF Laboratories, Welwyn Garden City, for providing burimamide, 2-methyl histamine and 4-methyl histamine used in this study.

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*(Received July 21, 1976.*
*Revised August 31, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 353–366

# THE DISTRIBUTION AND METABOLISM OF ARACHIDONIC ACID IN RABBIT PLATELETS DURING AGGREGATION AND ITS MODIFICATION BY DRUGS

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1 Gas chromatographic and radio-isotope labelling techniques have been used to establish the origin of the arachidonic acid used by the platelet cyclo-oxygenase for the synthesis of pro-aggregatory prostaglandin endoperoxide derivatives.

2 Measurements of total platelet arachidonate content indicated that more than 95% is esterified in the phosphatide fraction of the cells.

3 During aggregation by collagen or thrombin as much as 80% of the total platelet arachidonate may be liberated and transformed by the platelet enzymes into hydroxyacids and other more polar compounds.

4 The phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol fractions are major sources of the arachidonate thus used.

5 Indomethacin, which prevents platelet aggregation by inhibiting the cyclo-oxygenase, did not affect this release of arachidonate from the phosphatides but did prevent the transformation of arachidonate to endoperoxide derivatives.

6 Mepacrine, a drug which possesses weak anti-phospholipase activity in platelets, also prevents aggregation by collagen or thrombin, but seems to do so by preventing substrate release from the phosphatide fraction.

7 It is suggested that phospholipase $A_2$ plays a key role in the initial events during platelet aggregation induced by collagen.

## Introduction

Platelets contain at least two enzyme systems which oxygenate arachidonic acid (as well as some other polyunsaturated fatty acids) and a summary of these pathways is depicted in Figure 1. One of the transformations is catalysed by a cytoplasmic lipoxygenase (Hamberg & Samuelsson, 1974; Nugteren, 1975) and results in the formation of a lipid hydroperoxide, and ultimately a hydroxy acid named (by Hamberg & Samuelsson) 'HETE'. The other more complex pathway is catalysed by a membrane bound cyclo-oxygenase complex which in the first instance transforms arachidonate to the prostaglandin endoperoxide intermediates $G_2$ and $H_2$ (Hamberg, Svensson, Wakabayashi & Samuelsson, 1974; see also Nugteren & Hazelhof, 1973). These endoperoxides may be further metabolized either to prostaglandins, to a 17-C hydroxy acid ('HHT') or to thromboxane (TX) $A_2$ or $B_2$ (Hamberg & Samuelsson, 1974; Hamberg, Svensson & Samuelsson, 1975). This latter pathway is of especial interest; $TXA_2$ is a potent but highly unstable compound (half-life at 37°C about 32 s) which causes 5-hydroxytryptamine release from platelets and irreversible aggregation, in nanogram quantities. Large amounts of $TXA_2$ (assayed by its stable metabolite, $TXB_2$) are formed during aggregation by various agents, and it represents a considerable proportion of total prostaglandin $G_2$ metabolism (Hamberg & Samuelsson, 1974). As a product of the cyclo-oxygenase pathway, $TXA_2$ formation is blocked by aspirin-like drugs (Vane, 1971) thus providing an explanation for the observation that aspirin prevents second phase platelet aggregation (O'Brien, 1968). All the available evidence points to a major role for $TXA_2$ in irreversible platelet aggregation induced by collagen, thrombin and probably other stimuli.

In order to generate $TXA_2$ the platelet requires arachidonic acid. Platelets aggregate in the presence of

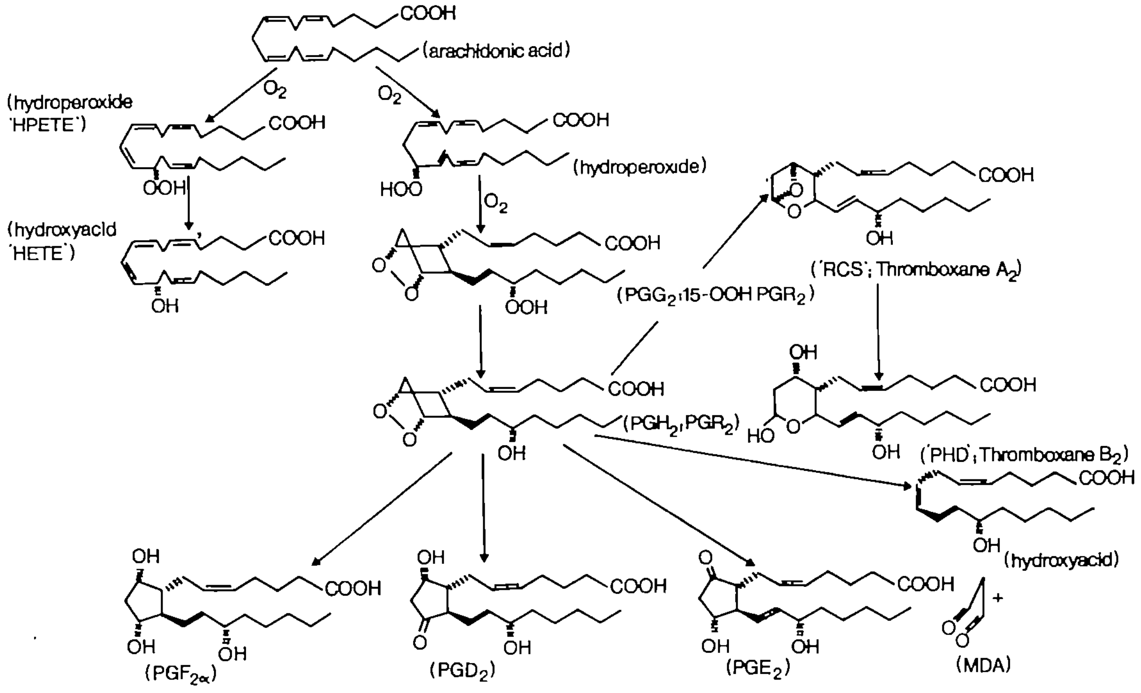

**Figure 1** A summary of the known pathways by which arachidonic acid is oxygenated in platelets. See text for references.

this fatty acid (Vargaftig & Zirinis, 1973; Silver, Smith, Ingerman & Kocsis, 1973) but 'spontaneous' aggregation of unstimulated platelets does not occur since the level of free arachidonic acid is apparently very low (Marcus, Ullman & Safier, 1969). Some preliminary reports by ourselves (Flower, Blackwell & Parsons, 1975) and other workers (Schoene & Iacono, 1975; Bills & Silver, 1975) suggested that several aggregating agents trigger cyclo-oxygenase activity by releasing arachidonic acid from the platelet phosphatide fraction. In this paper we describe experiments designed to answer the following questions: how much arachidonic acid is there in platelets, and what is the chief intracellular store? What is the source of the arachidonate used during platelet aggregation? Is phospholipase involved in the mobilization of substrate, and if so, what effect do phospholipase inhibitors have on platelet aggregation?

Whilst this manuscript was in preparation, Bills, Smith & Silver (1976) have published experiments indicating that phosphatidylcholine and phosphatidylinositol are important sources of the arachidonate used during aggregation of human platelets.

## Methods

### *Preparation of platelet rich plasma (PRP)*

Male New Zealand white rabbits were anaesthetized with pentobarbitone sodium (25–35 mg/kg). Blood was withdrawn from a cannula placed in the common carotid artery and collected in 10 ml tubes containing 1 ml of 3.8% (w/v) tri-sodium citrate. After mixing the blood was centrifuged at 250 × *g* for 15 min in a bench centrifuge and the PRP removed.

### *Measurement of platelet aggregation*

Platelet aggregation was measured by continuous recording of light transmission through PRP using a Born aggregometer (Born, 1962). Small (10–20 µl) volumes of solutions containing inhibitory drugs or aggregating agents were added to 1 ml aliquots of PRP in siliconized glass cuvettes. Continuous magnetic stirring was used to ensure adequate mixing and to prevent platelet sedimentation.

### *Preparation of collagen*

Acid soluble calf-skin collagen (type IV, Sigma) was homogenized in Tris-saline (pH 7.4) using a motor driven pestle. Suspensions were stored frozen, and thawed in a water bath for 30 min before use.

### *Preparation of washed platelets*

PRP was centrifuged at 300 × *g* for 20 min to sediment the platelets. The platelet-poor plasma was decanted and the platelets gently resuspended in Tyrode solution or Tris-saline (134 mM NaCl, 15 mM Tris-HCl, 5 mM D-glucose) giving an approximate final concentration of $10^8$ platelets/ml.

### *Extraction of lipids from platelets*

Aggregated or control samples (1 ml) of platelets were tipped quickly into 10 ml methanol, cooled with dry ice and the mixture stirred for 10 minutes. Chloroform (20 ml) was added to bring the chloroform/methanol ratio to 2:1 and after shaking, the mixture was placed in the refrigerator (2–4 °C) for 1 h to extract the lipids. The platelet extract was then filtered to remove precipitated protein and concentrated under vacuum in a rotary evaporator. The resulting lipid mixture was used for analysis and assay.

### *Separation into 'neutral' and 'polar' lipid classes*

Lipid extracts dissolved in chloroform were separated on 1–3 g (depending on the amount of lipid material) columns of activated silicic acid. Seven column volumes of chloroform eluted the neutral lipids (mono, di, tri-glycerides, fatty acids, hydroxy acids, cholesterol and its esters), and seven column volumes of methanol eluted the polar lipids (all phospholipids and lyso-derivatives, as well as cyclo-oxygenase products).

### *Hydrolysis of lipid fractions to yield fatty acid methyl esters*

In order to estimate the arachidonate content of phosphatides and neutral lipid esters a hydrolytic step must be included in the assay procedure. This was accomplished by the use of the boron tri-fluoride technique of Metcalfe, Schmitz & Pelka (1966). To the dried lipid extract was added 0.4 ml of 0.5 N NaOH in methanol. This was heated in boiling tubes for 10 min at 100°C in a water bath. The boron tri-fluoride solution (14% w/v) in methanol (0.5 ml) was added and the mixture boiled for 2 minutes. After cooling a saturated solution of NaCl was added until an oily layer separated. Hexane (1 ml) was added, the mixture shaken and the top layer containing fatty acid methyl esters removed for assay.

### *Gas chromatographic assay of arachidonate*

Hydrolysed lipid extracts (prepared as above) or the free acid (after methylation with diazomethane) were assayed directly with a Pye Unicam series 104 gas chromatograph. The column used was a 150 cm glass (outside diameter 0.6 cm) packed with 10% DEGS on Gas-chrom Q. A flow rate of 40 ml $N_2$/min was used. At an oven temperature of 185 °C, authentic methyl arachidonate was easily detected by the flame ionization detector (set at 250 °C) having a retention time of approximately 18 minutes. All measurements were made isothermally. Estimation of unknown amounts of arachidonate were made by comparing the (peak height × retention time) factor with a standard curve. Labelled arachidonate was used to correct for losses during extraction.

### *Pulse labelling of platelet suspensions with [1-$^{14}$C]-arachidonate*

Aliquots of washed platelets or in some cases PRP containing about $10^9$ platelets/ml were incubated with 1 µCi [1-$^{14}$C]-arachidonate for 1 h at room temperature. After washing off the excess label, the platelets were resuspended in Tyrode solution giving a final concentration of about $10^8$ platelets/ml. After a further 30 min incubation (designed to reduce the concentration of free arachidonate), the labelled platelet suspensions were exposed to aggregating agents or drugs, and extracted as already described.

### *Separation of phospholipids*

For determination of the amount of arachidonate (labelled or unlabelled) in individual phosphatides, the crude platelet lipid extract was first separated into neutral and polar lipids by column chromatography as described. The polar fraction was further separated by thin layer chromatography (t.l.c.) on preparative silica gel plates (250 µm layer). Two solvent systems were used: (I) chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1), and (II) chloroform/methanol/12 N ammonia/water (70:30:4:1). The $R_F$ values (× 100) for *authentic* phospholipids were as follows: solvent system I: sphingomyelin (SPH) and lysophosphatidylcholine (LPC), 7.6; phosphatidylcholine (PC), 16.0; phosphatidylinositol (PI), 26.2; phosphatidylserine (PS), 30.7; phosphatidylethanolamine (PE), 47.4. Solvent system II: LPC, 7.2; PS, 14.5; PI, 20.0; SPH, 21.8; PC, 31.5; PE, 48.4. The rank order of $R_F$ values found by us corresponds with those previously published for these solvent systems (Kates, 1972; Cohen & Derksen, 1969).

Phospholipid zones on the developed chromatograms were visualized by brief exposure to iodine vapour. The zones were eluted and hydrolysed with boron tri-fluoride for estimations of the arachidonate content, or in the case of the radio-isotope experiments the radioactivity in each zone was estimated by liquid scintillation counting.

### *Enzymatic hydrolysis of phospholipids*

To determine the position of the radioactive fatty acid, samples of some phospholipids were hydrolysed with the specific hydrolytic enzyme phospholipase $A_2$. Extracts containing the phosphatide were dissolved in Tris buffer (100 mM, pH 7.8) containing 5% Triton-X, 20 mM $CaCl_2$ and 100 mM NaCl. Pig pancreas phospholipase $A_2$ (100 µg enzyme) was added and the mixture incubated at 37°C for 1 hour. The radioactivity was extracted with chloroform/methanol (2:1), excess solvent dried off *in vacuo* and the sample spotted quantitatively onto a silica gel t.l.c. plate (100 µm layer) and developed to a distance of 10 cm in chloroform/methanol/acetic acid (90:5:5). Phosphatides and their lysoderivatives remain at the origin and fatty acids run at the solvent front. The amount of radioactivity in each zone was estimated by liquid scintillation counting.

### *Measurement of organic phosphorus*

Organic phosphorus was measured by the direct colorimetric method of Raheja, Kaur, Singh & Bhatra (1973), using PC as a standard.

### *Separation of radioactively labelled cyclo-oxygenase and lipoxygenase products by t.l.c.*

Two types of procedure were used. During aggregation 1 ml aliquots of labelled platelet suspension were tipped into beakers containing 10 ml ethanol +1% stannous chloride (w/v) to reduce the prostaglandin endoperoxides to prostaglandin $F_{2\alpha}$. Extraction then proceeded in the same way as already described and the constituents were separated by t.l.c. on a silica gel plate (100 µm layer) using ethyl acetate/*iso*-octane/acetic acid/water (11:5:2:10) as a developing solvent. In this system phosphatides remained at the origin, prostaglandin $F_{2\alpha}$ had an $R_F$ value (×100) of about 18, and arachidonic acid an $R_F$ of about 72. Zones containing radioactivity were detected either with a Panax radiochromatogram scanner, or by scraping off the chromatogram in 0.5 cm zones and estimating the radioactivity in each zone by liquid scintillation counting.

No stannous chloride was used for the second method of assaying these products; after extraction in the usual way, the mixture was separated by t.l.c. as above. The phospholipid zone was removed from the origin and the remainder of the radioactive products eluted from the plate with chloroform:methanol (2:1). When the solvent was evaporated the residue was redissolved in ether, the products methylated with ethereal diazomethane and subsequently rechromatographed on t.l.c. using ethyl acetate/*iso*-octane/water (50:100:100) as a developing solvent. Methyl arachidonate has an $R_F \times 100$ value of 81.7 in this system, methylated prostaglandins and other polyoxygenated products remain at the origin and methyl esters of hydroxy acids have an $R_F \times 100$ value of about 43 (HHT) and 51.6 (HETE). Zones containing radioactivity were detected and measured as before.

### *Preparation of 2-([1-$^{14}$C]-oleoyl) phosphatidylcholine*

PC was prepared from fresh egg yolks and purified by column chromatography (Saunders, 1957), monitored by t.l.c. LPC (1-acyl-glycerylphosphorylcholine) was generated by the action of venom phospholipase $A_2$ on the PC. To 184 mg PC in 50 ml diethyl ether was added 1 ml borate buffer (0.1 M; pH 7.4) containing 4.4 mg *Crotalus adamanteus* venom (Sigma) and 7 mg calcium acetate (dried). The flask was shaken slowly at 25°C. A white gelatinous precipitate adhering to the flask appeared within 30 min and the shaking was continued for 3 hour. The ethereal supernatant was discarded and the precipitate was washed twice with 10 ml ether, transferred to a small tared flask with chloroform/methanol (1:1), evaporated to dryness, dried in a vacuum desiccator and weighed. The residue (145 mg) was dissolved in 5 ml ethanol + 2 ml dichloroethane and stored at −20°C under nitrogen. It was shown by t.l.c. to consist of LPC with no detectable contamination with lecithin.

The biosynthesis of the specifically labelled PC was carried out as described by Robertson & Lands (1962). The reaction flask contained 250 µCi of [1-$^{14}$C]-oleic acid (57 mCi/mmol), 2 mg carrier oleic acid and 20 mg LPC. Organic solvents were removed under nitrogen, phosphate buffer (150 ml; 0.1 M; pH 7.5) was added and the mixture sonicated. When the lipids were dispersed the following reagents were added: 200 µmol adenosine triphosphate (ATP) (in a small volume of water, pH adjusted to 6.5), 200 µmol $MgCl_2$, 2 µmol CoA (in water, pH 6.5) and one third (3 ml) of a suspension in 0.25 M sucrose of the microsomes from 11 g of rat liver. Incubation was carried out at 37°C with gentle shaking. At 30 min and 120 min further equal amounts of ATP, $MgCl_2$, CoA and microsomes were added. After 3 h incubation the mixture was extracted with chloroform/methanol (2:1) and the chloroform phase dried over anhydrous $MgSO_4$. The extract was concentrated and applied to a column of 16 g silicic acid + 4 g Hyflo slurried with 10% (v/v) methanol/chloroform. Unreacted fatty acids were eluted with 100 ml of the same solvent. This was followed by 40% methanol/chloroform to elute the labelled phospholipid. The radioactivity of 50 ml fractions was monitored by liquid scintillation counting and fractions corresponding to the radioactive peak were combined. T.l.c. and radioactive scanning and counting showed that the product was [$^{14}$C]-PC having a radiochemical purity of about 99% and containing about 70% of the initial activity. Hydrolysis of this product with the snake venom enzyme showed that about 97% of the radioactivity was in the 2-acyl position (assuming that the enzyme was absolutely specific for the 2-position).

### *Assay of platelet phospholipase $A_2$ activity*

Washed platelet suspensions were lysed by freeze-thawing three times, and the crude preparation thus obtained was used as a source of enzyme. Each reaction set contained the following components: 0.2 ml lysed platelet suspension (about 30 mg/ml protein) homogenized in pH 7.4 Tris buffer (100 mM) containing 0.5% Triton-X; 0.1 ml 200 mM $CaCl_2$; 0.1 ml labelled phosphatide solution (about 50,000 ct/min); 0.1 ml buffer or inhibitory drug. After a thorough mixing the samples were incubated for 1 h at 37°C, the lipids extracted with chloroform-methanol (2:1) and separated by t.l.c. on silica gel plates (100 µm layer) developed in chloroform/methanol/acetic acid (90:5:5). Phosphatides remain on the origin, whereas liberated (radioactive) fatty acids migrate to the solvent front. In one set of experiments labelled phosphatide (0.5 µCi) was incubated with 5 ml aliquots of PRP directly. Two samples one of which contained 50 µM mepacrine were aggregated with thrombin, whilst a third sample served as a non-aggregated control. After 15 min treatment with the aggregating agents, the samples were extracted and the hydrolysis of the labelled phospholipid measured as described.

### *Measurement of protein*

This was done by the biuret method of Gornall, Bardawill & David (1949).

### *Scintillation counting procedures*

The radioactivity in the samples was estimated with a Beckman LS-150 liquid scintillation counter. The scintillation counting fluid used was Beckman 'Cocktail D' (5 g PPO and 100 g naphthalene per litre dioxane). All samples were counted such that $2\sigma \ngtr 0.5\% \bar{x}$. All ct/min values were converted to d/min by the AES ratio method.

The following drugs and chemicals were used: indomethacin (Merck); mepacrine (May and Baker); [1-$^{14}$C]-oleic acid, 57 mCi/mmol, and [1-$^{14}$C]-arachidonic acid, 58 mCi/mmol (Radiochemical Centre, Amersham); glass backed t.l.c. plates 2 mm layer of Kieselgel 60 (Merck); Triton-x 100 and *Crotalus adamanteus* venom (Sigma); pig pancreas phospholipase $A_2$ (Boehringer Mannheim G.m.b.H.); Silicic acid type I 60–200 mesh (Sigma). All other lipid standards were obtained from Sigma or Koch-Light. All other reagents (buffer salts, chromatography solvents) were analar grade or the highest purity available.

## Results

### *Location and content of arachidonic acid in rabbit platelets*

In two experiments the total arachidonate content of known numbers of washed platelets was measured in

**Table 1** Distribution of arachidonic acid in different platelet lipid fractions

| | | | *Distribution (mol %)* | | |
|---|---|---|---|---|---|
| *Experiment* | *Platelet count* | *Total arachidonate* | *Free acid* | *Neutral esters* | *Phosphatides* |
| 1 | $2.60 \times 10^8$ | 6.00 µg | ~1.0 | <1.0 | 96.8 |
| 2 | $4.07 \times 10^8$ | 6.65 µg | ~1.0 | <1.0 | 97.1 |

**Table 2** Distribution of phospholipid phosphorus and arachidonic acid between platelet phosphatide pools (one experiment)

| *Phosphatide pool* | *Phospholipid arachidonate pmol %* | *Phospholipid P g atom %* |
|---|---|---|
| Sphingomyelin | Trace | Not assayed |
| Lysophosphatidylcholine | Trace | Not assayed |
| Phosphatidylserine | 13.12 | 3.44 |
| Phosphatidylethanolamine | 46.65 | 25.46 |
| Phosphatidylinositol | 28.43 | 21.80 |
| Phosphatidylcholine | 11.80 | 49.30 |

the three lipid fractions (free acid, neutral esters, phosphatides) after silicic acid column chromatography and hydrolysis with $BF_3$ in methanol. The results are shown in Table 1, and give an approximate range of 16–23 ng of arachidonate per $10^6$ platelets. The distribution studies indicate that 97% of the arachidonate is located in the platelet phosphatide fraction. Rabbit (platelet-poor) plasma itself seems to contain considerable amounts of arachidonate and it is important to ensure that platelets are washed carefully when this type of experiment is undertaken.

Table 2 shows the distribution of phospholipid arachidonate and phospholipid phosphorus between the different phosphatide classes after separation of the polar fraction of the column effluent by t.l.c. In the initial experiments, we experienced great difficulty in obtaining reproducible separation of the different classes of phospholipids, and there appeared to be little correspondence between the platelet extract and the authentic phospholipid markers. The situation was further complicated by the finding that hydrolysis of phosphatides sometimes occurs in solvent system I (which contains acetic acid). The reason for the discrepancy between the 'standards' and the platelet extract was probably due to excess loading of the t.l.c. plates and the presence of other polar materials which tended to retard the migration of the phosphatides. In practice we overcame this by eluting the iodine-positive zones and re-running them separately in the same solvent system. This procedure resulted in a more pure phospholipid preparation and a good correspondence with the authentic standards.

Table 2 shows that SPH and LPC contain virtually no arachidonic acid; approximately half the platelet phospholipid arachidonate resides in the PE fraction, approximately one-third in the PI fraction, and the remainder is distributed equally between the PC and PS fraction. Amongst the major phosphatide stores PC represents about half of the cell phosphatides on a molar basis, PE and PI together represent just less than half, with PS comprising only a few moles per cent.

### *Effect of collagen and thrombin on platelet phospholipid arachidonate content*

Table 3 is a summary of the experiments in which platelet phospholipid arachidonate was assayed by g.l.c. before and after aggregation by collagen or thrombin. Because of the lengthy extraction-purification-assay techniques involved it was not possible to do sufficient experiments in a single group for statistical analysis, however, in all cases (except in experiment 9c) there was a marked decrease in the platelet phospholipid arachidonate content in both washed and PRP platelet suspensions after aggregation by either collagen or thrombin. This decrease of phospholipid arachidonate ranged from a fall of only 6.9% (experiment 9a) to a maximum of 85.5% (experiment 7), with a mean value of 41.6%. Whilst developing this technique we found that from time to time we obtained anomalous results; experiment 9c (Table 3) for example shows an apparent rise in phospholipid arachidonate after aggregation. This may have been due to a mishandling (and consequent hydrolysis) of the phospholipid sample during sample extraction.

**Table 3** Effect of aggregating agents on platelet phospholipid arachidonate. Summary of g.l.c. data

| | | *Platelet phospholipid arachidonate (µg)* | | | |
|---|---|---|---|---|---|
| *Experiment* | *Aggregating agent* | *Control* | *Aggregated* | *+Mepacrine (50 µM)* | *+Indomethacin (10 µM)* |
| 1 a (W)* | Collagen 100 µg | 6.41 | 2.24 | — | — |
| b (W) | Collagen 100 µg | 7.90 | 4.90 | — | — |
| 2 (W) | Collagen 100 µg | 6.01 | 2.36 | 6.31 | — |
| 3 (W) | Collagen 100 µg | 5.92 | 5.18 | 5.90 | — |
| 4 (W) | Collagen 100 µg | 8.41 | 6.19 | 7.80 | 6.52 |
| 5 (W) | Thrombin 0.25 u | 4.30 | 2.84 | — | — |
| 6 (W) | Thrombin 0.25 u | 5.63 | 4.69 | — | 4.81 |
| 7 (W) | Thrombin 0.25 u | 7.60 | 1.10 | — | 1.31 |
| 8 a (PRP) | Collagen 20 µg | 5.25 | 2.35 | — | — |
| b (PRP) | Collagen 20 µg | 5.85 | 3.35 | — | — |
| 9 a (PRP) | Collagen 20 µg | 5.80 | 5.40 | 7.00 | — |
| b (PRP) | Collagen 20 µg | 5.80 | 2.60 | 5.61 | — |
| c (PRP) | Collagen 20 µg | 8.20 | 9.40 | — | — |

* Arachidonate estimations made in washed platelets (W) or in platelet rich plasma (PRP).

### *Effects of mepacrine and indomethacin on platelet phospholipid arachidonate content*

In some experiments (see Table 3) mepacrine (50 µM) and indomethacin (10 µM) were tested. Indomethacin (3 experiments) had virtually no effect on the loss of arachidonate from the phospholipids, but mepacrine (5 experiments) definitely caused a retention of arachidonate within the phospholipid pool, in one instance (experiment 2) there was slightly more (~5%) arachidonate in the mepacrine-treated platelets than in the 'control' samples; again, poor technique may be responsible for this apparently anomalous result. Both indomethacin and mepacrine in the concentrations used here prevented aggregation of the platelets.

### *Uptake of [1-$^{14}$C]-arachidonic acid by platelets*

Excellent incorporation of [1-$^{14}$C]-arachidonic acid into rabbit platelets was observed. After analysis of the radioactivity in different lipid fractions it was found that >95% was incorporated into the phosphatide fraction, very small amounts (1–2%) were recovered as neutral lipid esters and the remainder of the label was recovered as free arachidonate. Time course studies indicated that uptake began almost immediately and was linear for as long as 1 to 2 hours. Further analysis of the phosphatide fraction by t.l.c. indicated that the radioactivity was largely associated with the PC, PS and PI fractions with smaller amounts recovered from the PE zone. In order to establish the positional labelling of one radioactive phosphatide (PC), this zone was eluted and incubated with pancreatic phospholipase $A_2$. After reaction the mixture was extracted and rechromatographed on t.l.c. using chloroform/methanol/acetic acid (90:10:2) as a developing solvent. More than 98% of the radioactivity was liberated indicating that virtually all the label was esterified in the 2′-acyl position. When the liberated fatty acid was recovered, methylated and rechromatographed in hexane/diethyl ether/acetic acid (50:50:2) on t.l.c. plates impregnated with 10% silver nitrate, all the radioactivity co-chromatographed with authentic methyl arachidonate indicating that no change in the chain length or degree of unsaturation of the labelled substrate had occurred, and thus it was still arachidonic acid.

### *Effect of aggregating agents and drugs on the distribution of [1-$^{14}$C]-arachidonate*

Table 4 shows the results in an initial series of experiments in which the platelet phospholipid [1-$^{14}$C]-arachidonate was measured before and after aggregation by collagen, and treatment with collagen in the presence of mepacrine. The incorporation of labelled substrate in these experiments was rather less than usual (33% as opposed to about 95%) but after aggregation there was a fall of 36.6% of phospholipid arachidonate compared with the control. In the platelets treated with mepacrine there was a decrease of only 15.3% indicating that mepacrine conserved the phospholipid arachidonate.

The next experiments were designed to find out which phospholipid pool(s) was depleted of [1-$^{14}$C]-arachidonate during aggregation. Figure 2 is a thin layer chromatogram in which the radioactivity in each phosphatide fraction was measured after separation in chloroform/acetone/methanol/acetic acid/water, (6:8:2:2:1), before and after aggregation by collagen. Only three zones of radioactivity were initially observed; however, upon re-chromatography (see previous section) one zone (first peak on left in Figure 2) was resolved into two separate peaks having mobilities corresponding to PC and PI. It appears that PC and PI in extracts tend to co-chromatograph even though the standards are quite well resolved. This 'rechromatography' technique was routinely employed in our experiments to obtain good resolution of the different classes. Figure 2 does show that there is a marked decrease in the combined PC/PI peak as well as in the PE peak; no incorporation of label into the SPH zone was detected and virtually no change in the PS pool was found. The slight 'shoulder' in the PS peak may have been a very small separate peak but was not further identified.

To obtain more quantitative information about the decrease in arachidonate content of each pool, further experiments were performed using rechromatography to obtain maximum resolution on t.l.c. Table 5 shows an analysis of two such experiments. The quantitative

**Table 4** Effect of collagen and mepacrine on platelet phospholipid arachidonate

| *Sample* | *% Radioactivity recovered in phosphatides (n = 5)* |
|---|---|
| Control | 33.76 (±1.19) |
| Aggregated (100 µg collagen) | 21.41 (±1.36) |
| Collagen + mepacrine (50 µM) | 28.61 (±1.70) |

Results are mean ± s.e. mean.

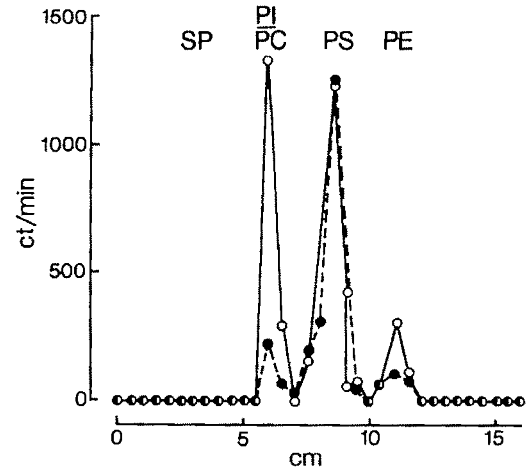

**Figure 2** Separation of labelled platelet phosphatides by thin layer chromatography (chloroform/acetone/methanol/acetic acid/water, 6:8: 2:2:1). SPH, PC, PS, PE and PI indicate the position of authentic sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol markers. (○) Control samples, (●) samples aggregated with 100 μg collagen. The plate was scraped into 0.5 cm zones and the radioactivity in each zone was estimated by liquid scintillation counting.

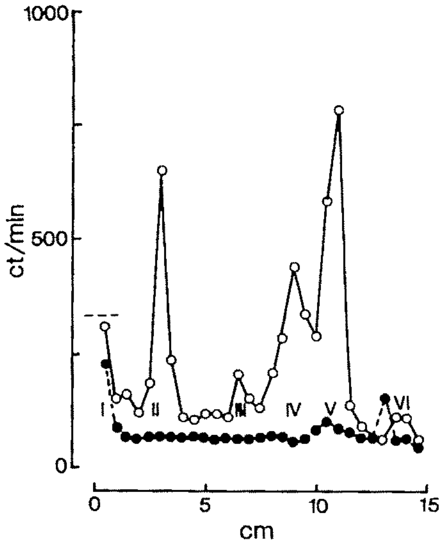

**Figure 3** Separation of radioactive arachidonate oxidation products by thin layer chromatography (ethylacetate/*iso*-octane/acetic acid/water, 11:5: 2:10) after treatment with 1% $SnCl_2$ in methanol. Peak I, labelled phosphatide fraction (off scale); II, prostaglandin $F_{2\alpha}$; III, unidentified; IV, hydroxyacid—HETE?; V, arachidonic acid; VI, unidentified. (○) Aggregated sample (collagen 100 μg); (●) non-aggregated control. The plate was scraped into 0.5 cm strips and the radioactivity in each estimated by liquid scintillation counting.

data thus obtained were similar to the qualitative data shown in Figure 2. The PC, PE and PI pools all showed large reductions but there was little change in the PS pool. In molar terms almost half the [1-$^{14}$C]-arachidonate released was derived from PC, PI contributing almost one-third and PE about one-fifth. A small increase in LPC was observed after aggregation.

### *Metabolic fate of the [1-$^{14}$C]-arachidonic acid liberated from phospholipids during aggregation*

To determine the ultimate metabolic fate of the arachidonic acid liberated during aggregation, thin layer chromatograms of lipid extracts of aggregated or control platelets were prepared. Figure 3 shows the distribution of radioactivity in 0.5 cm zones of a chromatogram developed in ethyl acetate/water/*iso*-octane/acetic acid (11:10:5:2). In this particular experiment stannous chloride (1% w/v) in methanol was added in a tenfold excess to the platelets when they had begun to aggregate. This manoeuvre should result in reduction of the endoperoxide intermediates to prostaglandin $F_{2\alpha}$ (see Hamberg & Samuelsson, 1974). Indeed, one large peak of radioactivity was

**Table 5** Distribution of [1-$^{14}$C]-arachidonic acid between platelet phospholipid fractions before and after aggregation (mean of two experiments)

| *Phosphatide fraction* | *pmol [1-$^{14}$C]-arachidonate* Control | Aggregated | Δ | *Decrease in pool size pmol %* Δ | *Fraction of total [1-$^{14}$C]-arachidonate contributed by each pool* |
|---|---|---|---|---|---|
| Sphingomyelin | Nil | Nil | Nil | Nil | Nil |
| Lysolecithin? | 8.27 | 13.47 | +5.20 | — | Nil |
| Phosphatidylserine | 23.80 | 21.72 | −2.80 | −8.75 | 7.21 |
| Phosphatidylethanolamine | 15.72 | 9.22 | −6.51 | −41.38 | 22.53 |
| Phosphatidylinositol | 28.64 | 20.37 | −8.28 | −28.89 | 28.66 |
| Phosphatidylcholine | 29.69 | 17.68 | −12.01 | −40.45 | 41.59 |

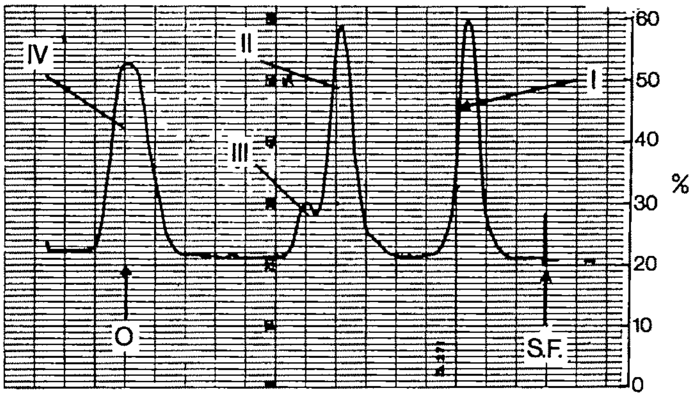

**Figure 4** Separation of radioactive methyl esters of poly-oxygenated products of platelet lipoxygenase and cyclo-oxygenase by thin layer chromatography (ethyl acetate/*iso*-octane/water, 50:100:100). The products formed have the same mobility as those formed by human platelets (Hamberg & Samuelsson, 1974). Peak I is unreacted arachidonate; the probable identity of the other products is: II, HETE; III, HHT; IV, cyclo-oxygenase products such as $TxB_2$ and small amounts of pr[o]staglandins. O=origin, SF=solvent front. Radioactivity was detected by a Panax radiochromatogram scanner.

found to co-chromatograph with prostaglandin $F_{2\alpha}$ (Peak II, Figure 3). Several other zones of radioactivity were also observed; Peak I was the labelled platelet phosphatide fraction (which is not on scale in the figure), peak III was unidentified but could conceivably be the hydroxyacid HHT, peak IV was almost certainly the hydroxyacid HETE—this compound was still formed from arachidonate in the presence of indomethacin, as are products of the lipoxygenase (Hamberg & Samuelsson, 1974). Furthermore, the polarity of the compound is exactly what one would expect for a hydroxyacid. Peak V co-chromatographed with arachidonic acid and Peak VI was unidentified.

**Table 6** Effect of aggregating agents and anti-aggregating drugs on the distribution and metabolism of radioactive arachidonate in rabbit platelets—Experiment 1

| | *pmol %* | | | |
|---|---|---|---|---|
| *Sample* | *Phosphatides* | *Free arachidonate* | *HETE* | *$TxB_2$+PGs* |
| Control | 93.68 | 2.71 | 0.86 | 2.74 |
| | 94.29 | 4.18 | 0.93 | 0.60 |
| Aggregated (collagen 100 μg) | 77.34 | 4.17 | 12.13 | 6.36 |
| | 80.47 | 2.75 | 6.25 | 10.25 |
| Collagen + indomethacin (10 μM) | 83.6 | 0.64 | 15.47 | 0.28 |
| Collagen + mepacrine (50 μM) | 92.91 | 2.01 | 0.46 | 4.61 |
| | 89.64 | 2.67 | 0.55 | 7.14 |

**Table 7** Effect of aggregating agents and anti-aggregating drugs on the distribution and metabolism of radioactive arachidonate in rabbit platelets—Experiment 2

| | *pmol %* | | | |
|---|---|---|---|---|
| *Sample* | *Phosphatides* | *Free arachidonate* | *HETE* | *$TxB_2$+PGs* |
| Control | 92.76 | 2.33 | 1.49 | 3.41 |
| | 92.43 | 3.21 | 1.98 | 2.38 |
| Aggregated (collagen 100 μg) | 74.74 | 5.31 | 8.07 | 11.88 |
| | 75.18 | 0.91 | 21.97 | 1.93 |
| Collagen + mepacrine (50 μM) | 83.60 | 3.38 | 2.11 | 10.9 |
| | 91.79 | 2.60 | 5.35 | 0.26 |

Treatment of platelets with methanolic stannous chloride proved to be a rather unreliable procedure for preparing platelet products for identification; often, sufficient stannous chloride extracted to interfere with the subsequent t.l.c. stages, and anyway chromatography of the free acids did not allow a (simple) complete separation of arachidonic acid and the hydroxyacid HETE. A far better method was to methylate the products after extraction and separate them by development in ethyl acetate/*iso*-octane/water (50:100:100). Figure 4 shows a radiochromatogram scan of the labelled products formed by rabbit platelets from labelled arachidonate derived from phosphatides. Four peaks are seen and these correspond very closely in their distribution to those formed by human platelets as published by Hamberg & Samuelsson (1974). The identity of each zone of radioactivity is based upon a comparison with the chromatogram published by these workers, pharmacological evidence (disappearance with indomethacin denotes it is a cyclo-oxygenase product) and in some cases comparison of $R_F$ values with authentic compounds. The zones of radioactivity have been identified as: I, arachidonic acid; II, HETE; III, HHT; IV, a mixture of polar cyclo-oxygenase products comprising small amounts of prostaglandins $E_2$ and $F_{2\alpha}$, a very large amount of another compound assumed to be $TXB_2$ (based on its behaviour in the solvent system, benzene/dioxan, 60:40) and smaller quantities of a very polar unidentified compound. In practice it was convenient to refer to peak IV (which remained on the origin) as 'cyclo-oxygenase products'. It is very difficult to separate the two hydroxy acids HHT and HETE but HHT was such a small component that it was ignored, the only radioactivity quantitated in this zone was HETE. The results which are now presented should be interpreted with these two factors in mind.

Tables 6 and 7 show a quantitation of the total phospholipid arachidonate metabolism. Because of the complexity of the assays only two experiments (in duplicate) were performed.

Experiment 1 (see Table 6) shows that in samples of non-aggregated platelets only very small amounts of HETE and cyclo-oxygenase products were found but these could increase some tenfold during collagen-induced aggregation, the label coming from the phospholipid fraction. The levels of free arachidonate did not increase, presumably because these samples were allowed to aggregate completely and thus use all the arachidonic acid available. Experiment 1 demonstrates that in the presence of indomethacin, there was still a loss of arachidonic acid from the phospholipid fraction but that this was not converted into the pro-aggregatory cyclo-oxygenase products, nor did the substrate accumulate since it was all transformed into HETE. Mepacrine had a completely different effect conserving the substrate within the phospholipid fraction, resulting in a diminished yield of both cyclo-oxygenase and lipoxygenase products, but especially the latter. Experiment 2 (Table 7) shows basically the same results as experiment 1 except that there was a larger degree of variation between the duplicates.

### *Effect of mepacrine and indomethacin on platelet aggregation*

Figure 5 shows an experiment in which collagen and arachidonic acid were used as aggregating agents, and mepacrine and indomethacin as inhibitors. Indomethacin (10 µM) blocked the aggregating activity of both arachidonic acid and collagen. Mepacrine, however, although it easily blocked the aggregation by collagen had no action on the velocity of the aggregation induced by arachidonate (which acts by direct conversion into pro-aggregatory endoperoxides) although it did reduce slightly the degree of aggregation. The concentration of mepacrine is apparently critical for this selective blockade of collagen-induced aggregation since at higher doses this drug also blocked arachidonate-induced aggregation, presumably by inhibition of the cyclo-oxygenase.

In two experiments the anti-aggregating ($IC_{50}$) concentration of mepacrine and indomethacin were measured against both arachidonate and collagen (Table 8). The $IC_{50}$ of indomethacin was almost the same against both agents suggesting a common mode of action. With mepacrine there was a clear difference the $IC_{50}$ against collagen being about half that required for an equivalent block of arachidonate-induced aggregation. Although this experiment strongly suggests separate modes of action for mepacrine and indomethacin it also illustrates that mepacrine is a poor selective inhibitor, except under rigidly controlled conditions of concentration.

### *Measurement of platelet phospholipase $A_2$ activity and the anti-enzyme activity of mepacrine and indomethacin*

By the use of the specifically labelled phospholipid enzyme assay described in the methods section, rabbit platelet phospholipase $A_2$ activity was easily detectable. Time course studies suggested that the reaction was autocatalytic or that some change in the nature of the enzyme was required, since a 'lag' phase was observed of about 10–20 min duration during which hydrolysis was small. This was succeeded by a period of more rapid hydrolysis which was linear for at least 2 hours. In practice, hydrolysis was measured over a period of 1 hour.

Table 9 shows the results of experiments to determine the hydrolysis after 1 h (expressed per mg platelet protein) and the anti-phospholipase activity of the two drugs. Whilst indomethacin had little effect (<3% inhibition) on the enzyme even in concentra-

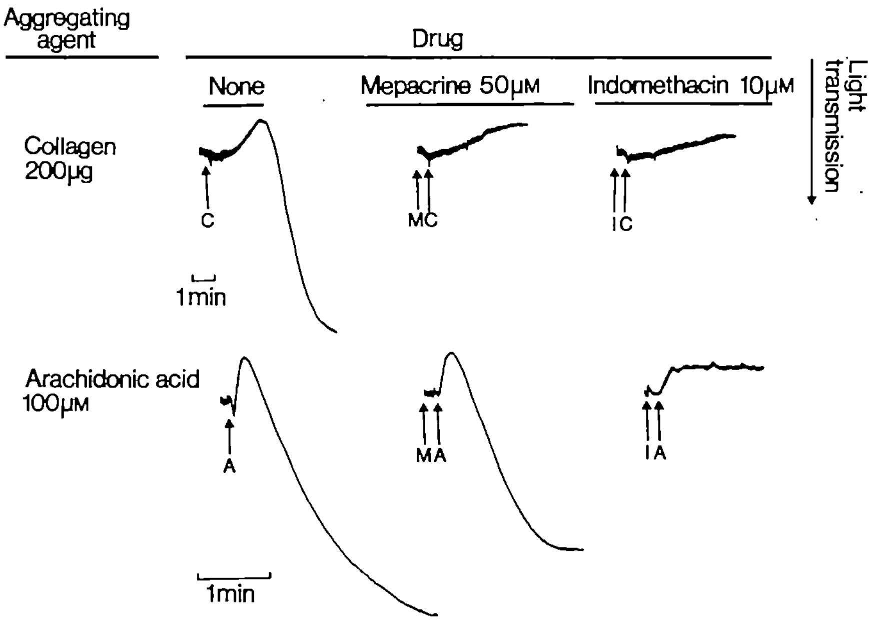

**Figure 5** Aggregometer tracings showing the differential blockade by mepacrine of collagen, but not arachidonate, induced aggregation.

**Table 8** Inhibition by indomethacin and mepacrine of platelet aggregation induced by collagen and arachidonic acid

| | $IC_{50}$ *(μM)* | |
|---|---|---|
| *Aggregating agent* | *Mepacrine* | *Indomethacin* |
| Arachidonic acid (100 μM) | 71 | 3.0 |
| Collagen (200 μg) | 34 | 3.5 |

**Table 9** Effect of mepacrine and indomethacin on rabbit platelet homogenate phospholipase $A_2$ activity

| *Sample (n=3)* | *pmol oleic acid liberated per mg protein (± s.e. mean)* | Δ% |
|---|---|---|
| Control | 39.20 (±3.12) | — |
| Mepacrine 50 μM | 37.33 (±2.28) | −4.76 |
| 500 μM | 23.73 (±1.52) | −39.53 |
| Indomethacin 10 μM | 39.57 (±1.68) | +0.94 |
| 100 μM | 38.29 (±2.64) | −2.32 |

**Table 10** Effect of thrombin (0.25 u/ml) on whole platelet phospholipase $A_2$ activity

| *Sample (n=3)* | *pmol oleic acid liberated per ml PRP/min (± s.e. mean)* | Δ% |
|---|---|---|
| Control | 25.33 (±5.90) | — |
| Thrombin aggregated | 65.05 (±6.35) | +256.8 |
| Thrombin + 50 μM mepacrine | 19.35 (±1.85) | −23.7 |

tions of 100 μM, mepacrine had a clear blocking action with an $IC_{50}$ value somewhat in excess of 500 μM. When aggregating agents such as collagen or thrombin were added to the platelet homogenate, no change in phospholipase $A_2$ activity was found.

The experiments in which the labelled phosphatide was added directly to the PRP, however, show a clear stimulation by the aggregating agent thrombin; phospholipase $A_2$ activity was suppressed (even below basal levels) by 50 μM mepacrine.

## Discussion

Two analytical techniques have been used to determine the origin, location and fate of the platelet arachidonic acid. By means of g.l.c. and lipid separation techniques, quantitative determinations of the amounts of arachidonate in different lipid pools of rabbit platelets have been made. These studies indicate that only trace amounts of free arachidonate are found in platelets, and that the amounts present as neutral lipid esters are also very low. Although 16–23 ng of arachidonate were found per $10^6$ platelets, about 97% of this was present in the platelet phosphatide fraction. These figures are very similar to estimates of the distribution of arachidonate in human platelets (Marcus *et al.*, 1969).

Following aggregation of platelets with collagen there was a consistent fall in the phospholipid arachidonate content, clearly demonstrating that this was the source of the substrate used for the synthesis of pro-aggregatory endoperoxide derivatives. This 'mobilization' of substrate which occurs during contact of the platelets with the aggregating agent collagen or thrombin is effectively blocked by mepacrine, but not by indomethacin.

Use of the radioactive tracer method for studying the movement of arachidonate during aggregation has disadvantages as well as advantages. Quantitation is much simpler when this technique is used and this (generally) enables larger sample sizes in each group. The use of radioactive arachidonate also enables all the products of the cyclo-oxygenase and lipoxygenase to be detected and measured; however, these assays are rather more complex. There are two theoretical disadvantages to this technique; one is that only lipid pools with relatively quick 'turnover' times are labelled and it is therefore not possible to detect the loss of fatty acids from non-labelled pools. A second objection is the 'isotope dilution' artefact; unlabelled arachidonic acid tends to dilute the labelled acid resulting in a low estimate of the final products formed.

Despite these objections, the results obtained by the two techniques show good corroboration. Labelled arachidonic acid is almost exclusively associated with the phospholipid fraction; in the case of the PC pool this acid is esterified in the 2′-acyl position and is not metabolically altered before incorporation. During contact with collagen the loss of arachidonate from the phosphatide fraction is clearly demonstrated, and analysis of the actual phospholipid pools indicates that this is derived chiefly from the PC, PI and PE fractions. These results are in general agreement with the figures published by Bills *et al.* (1976) for human platelets. Schoene & Iacono (1975) have also published some preliminary results using a tracer incorporation technique with human platelets. However, they concluded that the majority of the arachidonate liberated from platelet phosphatides is derived from the PI fraction.

A fundamental assumption underlying the use of the radioactive tracer experiments is that the incorporated labelled arachidonate behaves in an identical fashion to the endogenous arachidonate. Given that this is correct, a very important concept arises from examination of the data in Tables 2 and 5. Analysis of the data in Table 5 shows that both the PE and PC pools decrease by about 40% during aggregation, however, Table 2 shows that the PE pool contains almost four times as much arachidonate as the PC pool, so when interpreted in terms of the release of endogenous arachidonate PE actually contributes much more substrate. This calculation has been made for all phosphatide pools and is detailed in Table 11. PE contributes the most arachidonate during aggregation followed by PI then PC.

One interesting question is why the PS arachidonate is not liberated during aggregation. This could be because some phospholipase enzymes are specific for individual phospholipids, or because PS is a metabolically distinct pool.

When the oxidation products of the phospholipid arachidonate were examined they appeared to be almost identical to those formed from arachidonic acid by human platelets (Hamberg & Samuelsson, 1974). During aggregation the labelled arachidonate was transformed into two main classes of compound, a hydroxy acid fraction and another very polar fraction. The former is almost certainly HETE, a hydroxy acid formed by a soluble lipoxygenase enzyme in platelet cytoplasm (Hamberg & Samuelsson, 1974; Nugteren, 1975), and the latter is most probably $TXB_2$ together with smaller amounts of prostaglandins (Hamberg & Samuelsson, 1974). The amount of radioactivity found in each fraction was approximately equivalent (see Tables 6 and 7) except in the presence of indomethacin. This drug prevented the conversion of radioactive arachidonate into the cyclo-oxygenase products ($TXB_2$ and prostaglandins) and there was a concomitant increase in the amount of HETE formed. In the presence of mepacrine, there was a retention of arachidonate within the phosphatide fraction. This again confirms the results obtained with the g.l.c. and provides an explanation

**Table 11** Estimation of the total contribution of each phospholipid pool to the total amount of endogenous arachidonate released by platelets during aggregation

| *Phosphatide pool* | *% Decrease in* [1-$^{14}$C]-arachidonate during aggregation* | *Phosphatide arachidonate pool size, mol %*** | *Mol endogenous arachidonate released during aggregation* | *% Contribution from each endogenous pool* |
|---|---|---|---|---|
| Sphingomyelin | Nil | Nil | Nil | Nil |
| Phosphatidylcholine | 40.45 | 11.80 | 4.77 | 14.27 |
| Phosphatidylinositol | 28.89 | 28.43 | 8.21 | 24.56 |
| Phosphatidylserine | 8.75 | 13.12 | 1.14 | 3.41 |
| Phosphatidylethanolamine | 41.38 | 46.65 | 19.30 | 57.74 |

* Data from Table 5; ** Data from Table 2.

for the effect of mepacrine on platelet aggregation shown in Figure 5. The results suggest that collagen acts by liberating arachidonic acid from the platelet phosphatide fraction and that this step is mepacrine-sensitive. How is this effect of mepacrine brought about? One possibility is that the liberation of arachidonate from platelets is under the control of a phospholipase enzyme and that this is mepacrine-sensitive. In view of the positional labelling of the PC, this enzyme is presumably phospholipase $A_2$. The blocking action of mepacrine on this enzyme has already been reported by Vargaftig & Dao Hai (1972) and supported by other indirect evidence (Flower & Blackwell, 1976). Platelet phospholipase $A_2$ activity was easily detectable in lysed platelets but the reaction rate was slow, incubation periods of about 1 h being required to obtain good hydrolysis. This could be because the membrane bound enzyme obviously does not mix properly with a water soluble substrate. Mepacrine was found to block the enzyme activity but the concentrations required were some tenfold higher than those required to block collagen-induced aggregation; when the labelled phosphatide was incubated directly with PRP (i.e. whole platelets) hydrolysis was more rapid and was stimulated by the addition of thrombin, and blocked by lower concentrations of mepacrine. This discrepancy in activity might be explained on the basis of differences between lysed and whole platelets, or it could be that the inhibition of phospholipase is only an epiphenomenon and that mepacrine exerts its action by another distinct mechanism. A non-specific 'membrane stabilization' might be one such alternative mechanism although, as Figure 5 shows, platelets continue to respond to arachidonic acid when the effect of collagen is blocked. Even if mepacrine acts by blocking phospholipase $A_2$, it is unlikely that it will find a place as a selective inhibitor of this enzyme, for the $IC_{50}$ against the cyclo-oxygenase is only two fold greater than the $IC_{50}$ against phospholipase. Indomethacin, even at high concentrations, does not inhibit phospholipase $A_2$.

We believe this is the first study in which the metabolism of arachidonic acid has been followed from individual phosphatide pools to poly-oxygenated end products, and in which the two rate-limiting steps, phospholipase $A_2$ and the cyclo-oxygenase, have been shown to be targets for different types of drugs. As interest in poly-oxygenated fatty acid derivatives gathers momentum, studies such as the ones we report here will become valuable in determining the cellular origin of the substrate. In many respects, platelets are well suited for these studies as we have shown that there is very little arachidonate outside the phosphatide fraction; in other cells additional complications exist due to the fact that other lipid pools contain arachidonic acid (see Flower & Blackwell, 1976).

Reprint requests and correspondence to R.J.F.

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*(Received August 13, 1976.)*

*Br. J. Pharmac.* (1977), 59, 367–371

# REPEATED CHLORPROMAZINE ADMINISTRATION INCREASES A BEHAVIOURAL RESPONSE OF RATS TO 5-HYDROXYTRYPTAMINE RECEPTOR STIMULATION

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1 The hyperactivity syndrome produced in rats by administration of tranylcypromine (20 mg/kg i.p.) followed 30 min later by L-tryptophan (50 mg/kg i.p.) is generally considered to be due to increased 5-hydroxytryptamine (5-HT) functional activity. It is inhibited by chlorpromazine (30 mg/kg i.p.) injected 60 min before the tranylcypromine. However, chlorpromazine injection for 4 days either at a dose of 30 mg/kg once daily or 5 mg/kg twice daily results in an enhanced hyperactivity response to tranylcypromine and L-tryptophan administration 24 h after the final dose of chlorpromazine.

2 One injection of chlorpromazine (30 mg/kg) did not produce enhancement 24 h later and the inhibition of the tranylcypromine/L-tryptophan hyperactivity observed after acute chlorpromazine injection was seen if the rats were given tranylcypromine and L-tryptophan 1 h after the fourth chlorpromazine (30 mg/kg) dose.

3 Chlorpromazine (30 mg/kg) once daily or 5 mg/kg twice daily for 4 days resulted in rats displaying enhanced behavioural responses to the suggested 5-HT agonist 5-methoxy *N,N*-dimethyltryptamine (2 mg/kg) on day 5.

4 Chlorpromazine (30 mg/kg) once daily for 4 days produces a slight increase in brain 5-hydroxytryptamine (5-HT) concentration on day 5, but no difference in the rate of brain 5-HT synthesis or the rate of 5-HT accumulation after tranylcypromine and L-tryptophan administration.

5 There is some evidence that chlorpromazine blocks 5-HT receptors. It has also been observed that several other neuroleptic drugs do not produce enhanced 5-HT responses after repeated administration. It is suggested therefore that the enhanced behavioural response to 5-HT receptor stimulation following repeated chlorpromazine administration may be because this drug blocks 5-HT receptors.

## Introduction

Administration to rats of tranylcypromine and tryptophan increases the synthesis of brain 5-hydroxytryptamine (5-HT) and produces a hyperactivity syndrome (Grahame-Smith, 1971a) which has been used to investigate the way that various drugs alter the functional activity of 5-HT in rat brain (Green & Grahame-Smith, 1976). Acute chlorpromazine administration inhibits this behavioural response (Grahame-Smith, 1971b; Heal, Green, Boullin & Grahame-Smith, 1976) as does pretreatment with several other neuroleptic drugs (Heal *et al.*, 1976). Presumably this is because they block the dopaminergic system involved in this behavioural response to 5-HT receptor stimulation (Green & Grahame-Smith, 1974).

It was further demonstrated that repeated administration of several neuroleptic drugs enhanced the dopamine-induced locomotor response produced by injection of tranylcypromine and L-DOPA but that only chlorpromazine treatment enhanced the 5-HT-induced hyperactivity (Heal *et al.*, 1976). There is some evidence that chlorpromazine blocks 5-HT post-synaptic receptors (see Discussion section). It therefore seemed possible that the increased response was the result of receptor blockade in an analogous manner to the increased behavioural sensitivity of dopaminergic systems observed after repeated neuroleptic administration (Gianutsos, Drawbaugh, Hynes & Lal, 1974; Moore & Thornburg, 1975; Heal *et al.*, 1976). However, since it has been reported that chlorpromazine can alter the availability of tryptophan to the brain (Bender, 1976), the possibility existed that the increased response was the result of enhanced 5-HT synthesis and therefore a presynaptic change.

The reason for the enhanced 5-HT behavioural response has now been investigated and results suggest that it is a post-synaptic change and may reflect increased receptor sensitivity.

## Methods

Procedures for the experiments were as follows: chlorpromazine was either injected once daily (at a dose of 30 mg/kg) between 14 h 00 min–15 h 00 min or twice daily (10 h 00 min and 17 h 00 min) at a dose of 5 mg/kg. Control rats were injected with 0.9% w/v NaCl solution (saline) at the same time. Rats were given chlorpromazine on the schedules for 4 days. On day 5, 24 h after the final dose all groups were given tranylcypromine (20 mg/kg), and L-tryptophan (50 mg/kg) or 5-methoxy *N,N*-dimethyltryptamine (2 mg/kg) after a further 30 minutes.

Activity was measured in groups of 3 animals on LKB Animex activity meters (sensitivity and tuning: 30 μA) as described previously (Grahame-Smith, 1971a; Green & Grahame-Smith, 1974). Results were collected as movements/min and graphs show the mean of every 5 min period. Total movements over the 60 min period after either L-tryptophan or 5-methoxy *N,N*-dimethyltryptamine were measured and the results are given in the legend to each figure as the mean of the total movements in 60 min ± the s.e. mean (3 experiments). Differences between groups were calculated using Student's *t* test.

Brain tryptophan was measured by the method of Denckla & Dewey (1967) and brain 5-HT by the method of Curzon & Green (1970).

## Results

### *Effect of repeated chlorpromazine administration on the hyperactivity following tranylcypromine and L-tryptophan*

Rats were injected with saline or chlorpromazine (30 mg/kg) once daily for 4 days. On day five, 24 h after the last injection both groups were given tranylcypromine (20 mg/kg) and L-tryptophan (50 mg/kg) after a further 30 minutes. The enhanced activity response of rats to tranylcypromine and L-tryptophan following repeated chlorpromazine treatment was confirmed (Figure 1). Enhancement was also seen if the rats had been injected with chlorpromazine (5 mg/kg) twice daily (10 h 00 min and 17 h 00 min) for 4 days and challenged with tranylcypromine and L-tryptophan on day 5 as described above (Figure 1). This dose does not produce the severe sedation seen after the higher chlorpromazine dose.

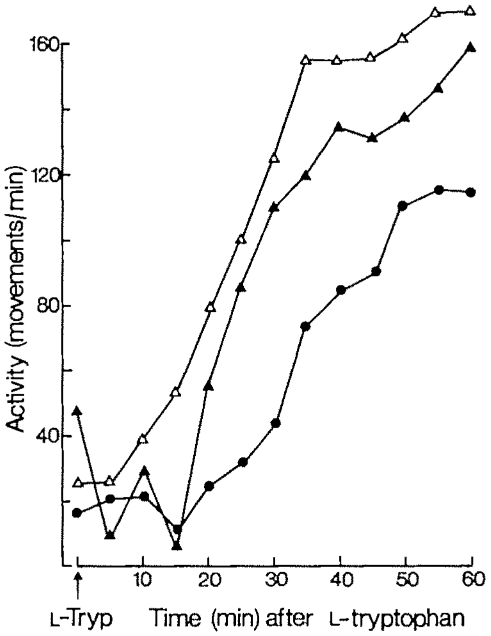

**Figure 1** Effect of repeated chlorpromazine administration on the hyperactivity response of rats to tranylcypromine and L-tryptophan. Rats were injected daily for 4 days with saline (●) or chlorpromazine 30 mg/kg (△) or twice daily with chlorpromazine 5 mg/kg (▲). Twenty-four hours after the final injection all groups were given tranylcypromine (20 mg/kg) with L-tryptophan (L-Tryp, 50 mg/kg) after a further 30 minutes. Graph shows the typical hyperactivity following L-tryptophan. Activity was measured as movements/min on Animex meters as described in the Methods section. Total movements during 60 min following L-tryptophan, control: 2960 ± 55; chlorpromazine (30 mg/kg) pretreated: 6593 ± 291; chlorpromazine (5 mg/kg x 2) pretreated: 4000 ± 357. All results are the mean ± s.e. mean of 3 observations and chlorpromazine-treated groups are significantly different from controls at a level of significance of $P < 0.01$ and $P < 0.025$ respectively.

No enhancement was seen if the animals were challenged with tranylcypromine and tryptophan 24 h after a single dose of chlorpromazine (30 mg/kg). Furthermore, when this dose was given daily for 4 days with tranylcypromine and L-tryptophan given 1 h after the final dose, the normal inhibitory action of chlorpromazine on the tranylcypromine/L-tryptophan hyperactivity was still observed.

### *Effect of repeated chlorpromazine administration on brain tryptophan and 5-hydroxytryptamine concentrations and the rate of 5-hydroxytryptamine synthesis*

An investigation was next made into the effect of the higher chlorpromazine dose on 5-HT biosynthetic mechanisms.

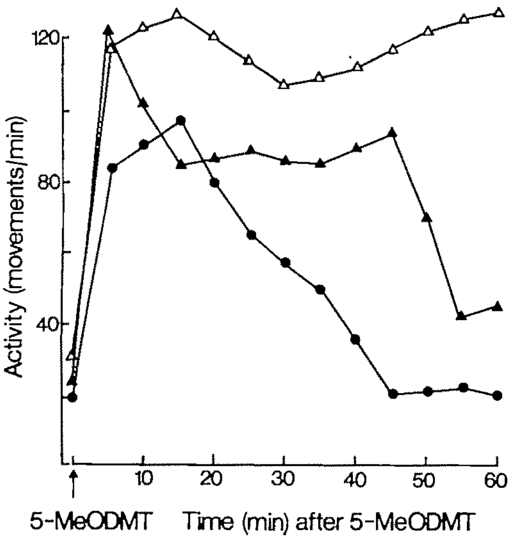

**Figure 2** Effect of repeated chlorpromazine administration on the hyperactivity response of rats to tranylcypromine and 5-methoxy *N,N*-dimethyltryptamine (5-MeODMT). Rats were injected daily for 4 days with saline (●) or chlorpromazine 30 mg/kg (△) or twice daily with chlorpromazine 5 mg/kg (▲). Twenty-four hours after the final injection all groups were given tranylcypromine (20 mg/kg) with 5-MeODMT (2 mg/kg) after a further 30 minutes. Graph shows the typical hyperactivity following 5-MeODMT. Activity was measured as in Figure 1. Total movements during 60 min following 5-MeODMT, control: 3700±509; chlorpromazine (30 mg/kg) pretreated: 8530±452; chlorpromazine (5 mg/kg × 2) pretreated: 5710±368. All results expressed as mean ± s.e. mean of 3 observations. Both chlorpromazine-treated results significantly different from control $P<0.01$.

Rats were injected with saline or chlorpromazine (30 mg/kg) daily for 4 days. On the fifth day 24 h after the last injection they were killed and brain tryptophan and 5-HT measured. Further groups were injected with saline or chlorpromazine using the same protocol but 24 h after the final injection they were given tranylcypromine (20 mg/kg) and killed 60 min later and brain 5-HT measured. The rate of 5-HT synthesis following monoamine oxidase inhibition was then calculated by the method of Neff & Tozer (1968).

No differences were observed in the brain concentration of tryptophan between the control and chlorpromazine-treated group although the steady state concentration of 5-HT following repeated chlorpromazine administration showed a slight increase (Table 1). The rate of 5-HT synthesis in both groups was the same (Table 1). The rise of brain 5-HT after chlorpromazine has been observed by Bender (1976) but the changes reported here are much smaller.

These results suggested that the enhanced responses seen after repeated chlorpromazine treatment were not due to a change in 5-HT synthesis and this was confirmed by the observation that 1 h after L-tryptophan, when the chlorpromazine-treated animals were displaying considerably greater activity (see Figure 1) the brain 5-HT concentrations in both groups were the same (Table 1).

### *Effect of repeated chlorpromazine administration on the behavioural response to tranylcypromine and 5-methoxy* N,N*-dimethyltryptamine*

Since the foregoing experiments showed that repeated chlorpromazine administration did not alter 5-HT biosynthetic mechanisms it seemed probable that post-synaptic changes were responsible for the enhanced 5-HT response following chlorpromazine. This was investigated by use of the suggested 5-HT agonist 5-methoxy *N,N*-dimethyltryptamine (5-MeODMT) which produces a qualitatively similar hyperactivity response to that seen after tranylcypromine and tryptophan, but with a different time course (Grahame-Smith, 1971b).

Rats were injected daily with saline or chlorpromazine as described above. Twenty-four hours after the final injection both groups were given tranylcypromine (20 mg/kg) followed 30 min later by 5-MeODMT (2 mg/kg). Chlorpromazine-treated animals showed a considerably enhanced response to 5-MeODMT (Figure 2) indicating altered post-synaptic responses following chlorpromazine.

Enhancement was also seen after the lower dose of chlorpromazine (5 mg/kg, twice daily, see above) (Figure 2).

## Discussion

The fact that repeated chlorpromazine administration does not alter the rate of 5-HT synthesis but does produce an enhanced response to a 5-HT agonist strongly indicates that repeated chlorpromazine administration produces enhanced 5-HT responses as the result of a post-synaptic change. This does not appear to be due to the dopamine receptor blocking action of this drug as several other neuroleptics examined (haloperidol, spiroperidol and α-flupenthixol) do not have this action (Heal *et al.*, 1976). It seems probable that the enhancement is due to blockade of 5-HT receptors by chlorpromazine. Bradley, Wolstencroft, Hösli & Avanzino (1966) using microiontophoretic techniques observed blockade of some 5-HT neurones by chlorpromazine and recently Von Hungen, Roberts & Hill (1975) demonstrated that chlorpromazine, but not

**Table 1** Effect of chlorpromazine on brain tryptophan and 5-hydroxytryptamine (5-HT) biosynthetic mechanisms

| | | | *Brain concentrations in μg/g brain tissue (wet wt.)* | |
|---|---|---|---|---|
| *Injected* | *Pretreatment* | *No. of observations* | *5-Hydroxytryptamine* | *Tryptophan* |
| — | Saline | 10 | $0.37 \pm 0.01$ | $2.98 \pm 0.32$ |
| — | Chlorpromazine | 10 | $0.43 \pm 0.01$* | $2.79 \pm 0.17$ |
| Tranylcypromine | Saline | 12 | $0.91 \pm 0.04$ | $18.40 \pm 1.51$ |
| + L-Tryptophan | Chlorpromazine | 12 | $0.95 \pm 0.03$ | $18.66 \pm 1.13$ |
| | | | | *Rate of 5-HT synthesis ($\mu g\ g^{-1}\ h^{-1}$)* |
| Tranylcypromine | Saline | 9 | $0.56 \pm 0.01$ | 0.19 |
| Tranylcypromine | Chlorpromazine | 8 | $0.60 \pm 0.01$ | 0.17 |

Results show brain tryptophan and 5-HT concentrations 24 h after 4 daily injections of chlorpromazine (30 mg/kg). The 5-HT accumulation in similarly treated rats given tranylcypromine (20 mg/kg) followed 30 min later by L-tryptophan (50 mg/kg) is also shown 60 min after the tryptophan administration. Finally the rate of 5-HT synthesis in a group of saline and chlorpromazine (30 mg/kg for 4 days)-treated rats on the 5th day has been measured. Rats were given tranylcypromine (20 mg/kg) and the 5-HT accumulation measured 60 min later. Subtraction of the mean control values from the value obtained 60 min after tranylcypromine gives the rate of synthesis in $\mu g\ g^{-1}\ h^{-1}$ (Neff & Tozer, 1968). Results show mean ± s.e. mean. The number of observations is noted.

* Different from saline-treated control, $P < 0.01$.

haloperidol, inhibited 5-HT-sensitive adenylate cyclase in the neonate rat, suggesting an action of chlorpromazine on the post-synaptic 5-HT receptor not shared by haloperidol.

While enhanced responses to dopamine receptor stimulation following repeated administration of drugs blocking the receptor now seem well established, the idea that 5-HT receptors can also exhibit this change has only recently been indicated. Klawans, D'Amico & Patel (1975) showed increased behavioural sensitivity to 5-hydroxytryptophan after chronic methysergide administration and Trulson, Eubanks & Jacobs (1976) have now demonstrated enhanced 5-HT post-synaptic responses following destruction of central 5-HT neurones with 5,7 dihydroxytryptamine. This was demonstrated by a shift in the dose-response curve of the behavioural responses to the 5-HT agonist 5-methoxy *N,N*-dimethyltryptamine. It should perhaps be noted however that Trulson *et al.* (1976) and I (unpublished observations) have been unable to demonstrate enhanced responses to 5-MeODMT following administration of *p*-chlorophenylalanine, the tryptophan hydroxylase inhibitor. This is at variance with the analogous situation in the dopaminergic system where treatment with the tyrosine hydroxylase inhibitor α-methyl *p*-tyrosine results in enhanced dopaminergic responses (Tarsy & Baldessarini, 1973; Dominic & Moore, 1969).

One interpretation of these findings would be that repeated chlorpromazine increases 5-HT receptor sensitivity. This is only observed however when the concentration of chlorpromazine in the brain has decreased since it was not apparent 1 h after the final administered dose, but was present 24 h later.

Whether the enhanced response is due to an alteration at the receptor itself or an alteration in some other neuronal system connected with the post-synaptic 5-HT receptor and which is seen as an increase in the 5-HT response, cannot be stated. However, the fact that haloperidol and several other neuroleptics do not produce this enhanced response argues strongly against the action of chlorpromazine on 5-HT responses being via its action on brain dopaminergic systems.

Since it has been demonstrated previously that the 5-HT hyperactivity syndrome is mediated via a post-synaptic dopaminergic system (Green & Grahame-Smith, 1974) the question arises as to why repeated administration of other neuroleptics, which demonstrably increase dopaminergic sensitivity (Heal *et al.*, 1976) does not result in increased 5-HT responses. No definitive answer can yet be given to this but it seems reasonable to suppose that transmitter interactions are modulatory and some alteration in the sensitivity of a dopaminergic system need not result in changes in 5-HT function.

I thank Mr M.R. Bloomfield and Miss A.F.C. Tordoff for technical assistance, Smith, Kline and French for tranylcypromine and Drs Trulson, Eubanks & Jacobs for allowing me to see their manuscript before publication.

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*(Received August 16, 1976.*
*Revised October 8, 1976.)*

*Br. J. Pharmac.* (1977), 59, 373–378

# INFLUENCE OF NEUROGLIAL TRANSPORT ON THE ACTION OF $\gamma$-AMINOBUTYRIC ACID ON MAMMALIAN GANGLION CELLS

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1 The effect of inhibiting the transport of $\gamma$-aminobutyric acid (GABA) by neuroglial cells on the depolarizing action of exogenous amino acids on isolated superior cervical ganglia of the rat was studied.

2 Transport (measured by uptake of [$^3$H]-GABA) was inhibited by (a) reducing external [Na$^+$] from 143 to 2 mM and (b) administering alternative carrier-substrates, 3-amino-*n*-butyric acid ($\beta$-aminobutyric acid, BABA) and ($\pm$)-nipecotic acid at a concentration of 1 mM.

3 All three procedures enhanced the depolarization produced by low concentrations of GABA ($\leq 10$ μM) but did not alter the maximum response, nor the response to 3-aminopropanesulphonic acid (a gabamimetic with low affinity for the neuroglial carrier).

4 It is concluded that the neuroglial uptake process can limit the action of exogenous GABA upon neurones, by reducing the interstitial GABA concentration.

## Introduction

The central inhibitory neurotransmitter $\gamma$-aminobutyric acid (GABA) depolarizes neurones in sympathetic ganglia (De Groat, 1970; Bowery & Brown, 1974), by increasing Cl$^-$ conductance (Adams & Brown, 1975). The neurones in this tissue are closely invested by neuroglial (satellite) cells, which possess a transport system capable of taking up exogenous radioactively-labelled GABA (Bowery & Brown, 1972; Young, Brown, Kelly & Schon, 1973). The question addressed in the present experiments is whether the effect of exogenous GABA on sympathetic neurones is modified by the activity of the adjacent glial carrier.

The results of our previous experiments are not clear on this point. Bowery & Brown (1974) and Adams & Brown (1975) observed no gross change in the depolarizing action of GABA on omitting Na$^+$ ions from the bathing medium, a procedure that inhibits carrier-mediated uptake of GABA by the glial cells (Bowery & Brown, 1972), but rather high concentrations of GABA were used in these experiments. On the other hand, Bowery, Brown, Collins, Galvan, Marsh & Yamini (1976: Figure 10) detected an increase in the depolarizing action of very low concentrations (< 10 μM) of GABA in Na$^+$-free solution.

In the present experiments the effect of reducing external [Na$^+$] on the depolarizing action of GABA has been assessed more carefully than hitherto. In addition, another method of reducing glial uptake of GABA, the application of false substrates ($\beta$-aminobutyric acid, BABA: Bowery *et al.*, 1976) and ($\pm$)-nipecotic acid (Krogsgaard-Larsen & Johnston, 1975) has been tested.

## Methods

Superior cervical ganglia with their attached pre- and postganglionic nerve trunks were isolated from Wistar rats (230–270 g, either sex) anaesthetized with urethane (1.5 g/kg). Potentials were recorded from desheathed superfused preparations as described by Brown & Marsh (1975). The superfusion fluid was Krebs-Henseleit solution of composition described previously (Bowery *et al.*, 1976), gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 25°C. In the present experiments ganglia were isolated the day before use and maintained overnight at 4°C: by allowing the initial demarcation potential to subside, this gives greater d.c. stability for high-gain recording, without altering the response of the ganglia to agonists or the

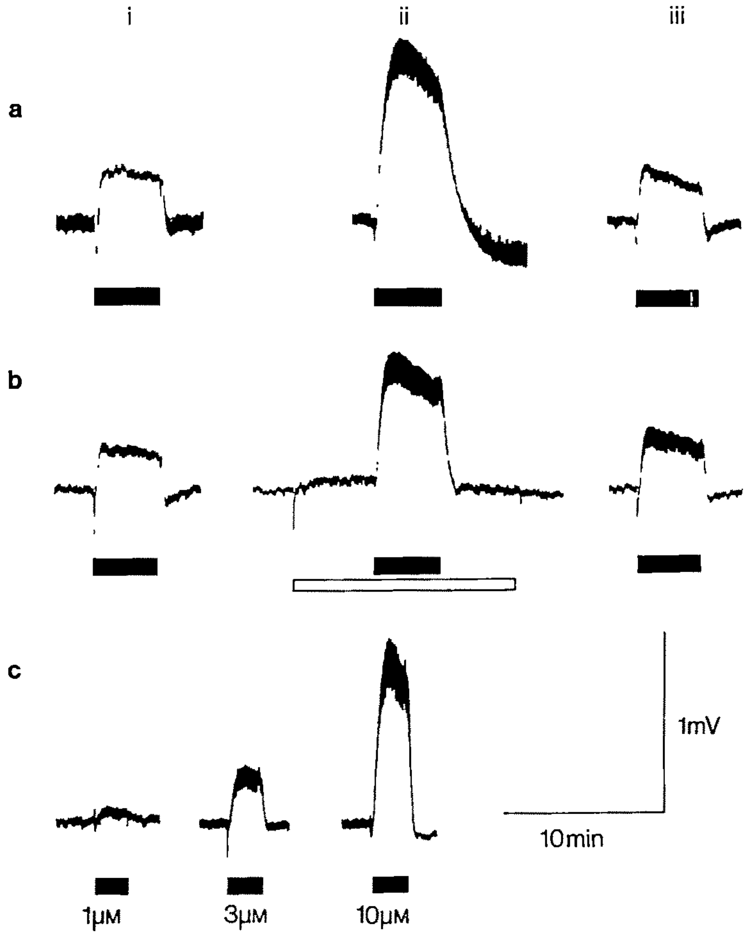

Figure 1 Depolarizations of an isolated ganglion of the rat produced by 4-min applications of 3 μM γ-aminobutyric acid (GABA, solid bars), (a) before (i), during (ii) and after (iii) exposure to low [Na⁺] solution and (b) before, during and after exposure to 1 mM β-aminobutyric acid (open bar). (c) Shows responses to calibrating concentrations of GABA applied for 2-min periods at the end of the experiment. Scale: 1 mV, 10 minutes.

activity of the GABA carrier when compared with freshly-dissected ganglia.

The normal $Na^+$ concentration of the superfusing fluid was 143 mM (118 mM as NaCl, 25 mM as $NaHCO_3$). Low (2 mM) $Na^+$ solution was prepared by replacing all but 2 mM $Na^+$ with $Li^+$, as LiCl and $Li_2CO_3$.

Saturable influx of [$^3$H]-GABA into isolated ganglia at 25°C was measured as described by Bowery *et al.* (1976), with 10 μM amino-oxyacetic acid added to the incubation medium to preclude metabolism of label. Inhibitors of uptake were added 10 min before adding substrate.

The following compounds were used: γ-aminobutyric acid (4-amino-*n*-butyric acid, GABA: BDH); β-aminobutyric acid (3-amino-*n*-butyric acid, BABA: Sigma); and 3-aminopropanesulphonic acid (3-APS: K. & K.). (±)-Nipecotic acid was synthesized by C. Cooksey according to the method of Freifelder (1963). Agonists were superfused over the preparation for 4 min at 30-min intervals, unless otherwise stated. The superfusion rate was ≃2 ml/minute. Dead time was noted using an air-interface, and exposure times corrected accordingly. Concentrations refer to those in contact with the ganglion.

## Results

Figure 1 illustrates one type of experiment carried out. In the upper trace (Figure 1a) constant low-amplitude depolarizing responses to a just-suprathreshold con-

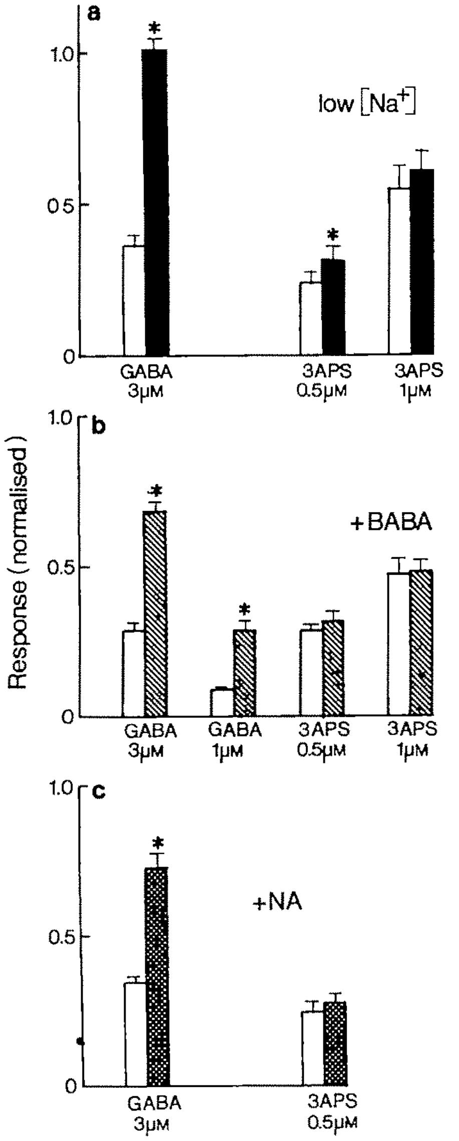

**Figure 2** Effects of inhibiting glial transport of γ-aminobutyric acid (GABA) upon the depolarizing action of exogenous GABA and 3-aminopropanesulphonic acid (3-APS). Responses to low doses of GABA and 3-APS are shown in normal solution (open columns), (a) in low [Na+] solution (closed columns), and during superfusion with either (b) 1 mM β-aminobutyric acid (BABA, hatched columns) or (c) 1 mM (±)-nipecotic acid (NA, cross hatched columns). Responses were normalized with respect to those produced by 10 μM GABA in normal solution (=1.0). Each column is the mean of 6 experiments except for experiments with (±)-nipecotic acid, where $n=4$. Vertical bars show s.e. means.

*Significant difference between means ($P<0.001$, Student's $t$ test); unmarked columns, $P>0.05$.

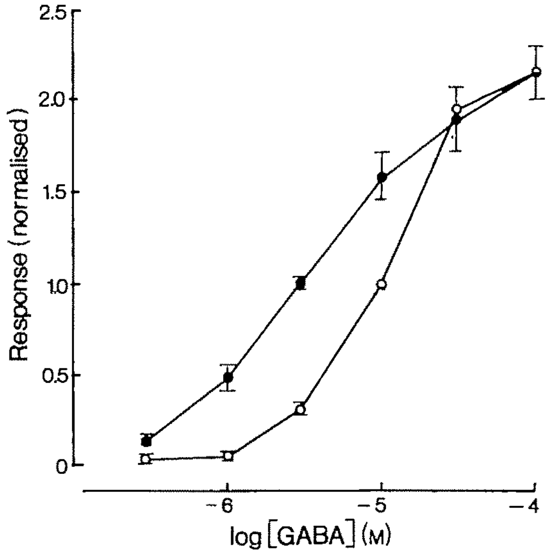

**Figure 3** Dose-response curves to γ-aminobutyric acid (GABA) measured in normal solution (○) and low [Na+] solution (●). Responses were normalized with respect to those produced by 10 μM GABA (=1.0) in normal solution to facilitate comparison between different ganglia. Each point is the mean of 6 determinations in different ganglia; bars=s.e. mean.

centration (3 μM) of GABA were obtained in normal Krebs solution containing 143 mM [Na+] (Record i). This solution was then replaced with one containing 2 mM [Na+] and 141 mM [Li+] (Record ii). After the electrode junction-potential changes had stabilized, 3 μM GABA now gave a response equal to that produced by 10 μM GABA in 143 mM [Na+] solution (Figure 1c). This potentiation was fully reversed on restoring Na+ ions (Record iii).

In the experiment illustrated in Figure 1b, a similar procedure was used except that 1 mM β-aminobutyric acid (BABA) was added to inhibit GABA uptake. The response to 3 μM GABA now became equivalent to that of 6 μM GABA in the absence of BABA.

A number of experiments of this type were performed, some using as an additional agonist 3-aminopropanesulphonic acid (3-APS). The results are summarized in Figure 2. There were consistent and substantial increases in the responses to 3 μM GABA in low-Na+, BABA and (±)-nipecotic acid solutions, but no corresponding increase in the equivalent-amplitude responses to 3-APS.

The effect of low-Na+ solution on the dose-response curve to GABA is shown in Figure 3. The control curve, in 143 mM [Na+] ($ED_{50}$ 11 μM) is comparable with that previously described by Bowery & Brown (1974) ($ED_{50}$ 12.5 μM). Low-Na+ solution increased the responses to low (≤10 μM) GABA concentrations, without altering the maximal response, thereby producing a non-parallel shift to the left and a new $ED_{50}$ of 3.5 μM.

### *Uptake inhibition*

The percentage inhibition of saturable [$^3$H]-GABA uptake at the substrate and inhibitor concentrations used in the present experiments are given in Tables 1 and 2.

At 3 μM [$^3$H]-GABA, saturable uptake was inhibited by 94% in 2 mM [$Na^+$], 70% in 1 mM (±)-nipecotic acid and 35% in 1 mM BABA. Inhibition in 2 mM $Na^+$ solution was constant at substrate concentrations from 0.3 to 100 μM, i.e., over the range in Figure 3.

## Discussion

In these experiments, procedures that reduced the uptake of [$^3$H]-GABA into ganglionic neuroglial cells (reducing external [$Na^+$] to 2 mM, or adding 1 mM β-aminobutyric acid (BABA) or (±)-nipecotic acid) strongly potentiated the depolarization of the ganglionic neurones produced by GABA. Available evidence suggests these two effects to be causally related for the following reasons:

(1) The same procedures did not appreciably affect the action of 3-APS. The concentrations of 3-APS used ($\leqslant 1$ μM) are so far below its $K_T$ for the GABA carrier (0.8 mM: Bowery, 1974) that it could be transported only at a very low rate. (A slight, though significant, augmentation at the lowest concentrations of 3-APS could be detected (see Figure 2): this is to be expected through the small additive effect of endogenous GABA released during uptake inhibition, Bowery *et al.*, 1976).

(2) The degree of potentiation showed some relationship to the amount of uptake inhibition produced by the three procedures. Potentiation by BABA is, perhaps, disproportionately great: this could be due to its weak direct agonist activity, which is just around threshold at the concentration used (1 mM: cf. Bowery *et al.*, 1976): (±)-nipecotic acid appears to

**Table 1** Uptake of [$^3$H]-γ-aminobutyric acid measured after 30 min incubation in 3 μM substrate solution at 25°C in the presence of 10 μM amino oxyacetic acid, expressed as the tissue/medium ratio (concentration of label in ganglion/concentration in incubation medium), in the absence and presence of transport inhibition.

| | *Tissue/medium ratio mean ± s.e. (n)* | | *Mean % inhibition* |
|---|---|---|---|
| Control | 7.99 ± 0.63 | (10) | 0 |
| 2 mM [$Na^+$] | 0.50 ± 0.05 | (8) | 94 |
| 1 mM (±)-nipecotic acid | 2.41 ± 0.11 | (4) | 70 |
| 1 mM β-aminobutyric acid | 5.19 ± 0.27 | (8) | 35 |

Uptake values are corrected for the minor non-saturable infiltration (Bowery *et al.*, 1976).

**Table 2** Effect of reducing [$Na^+$] from 143 mM to 2 mM on uptake of [$^3$H]-γ-aminobutyric acid (GABA) at different GABA concentrations

| | *Uptake velocity (μmol per l cell fluid/min)* | | |
|---|---|---|---|
| *μM GABA* | *143 mM [$Na^+$]* | *2 mM [$Na^+$]* | *% Inhibition* |
| 0.3 | 0.189 | 0.017 | 91.0 |
| 1.0 | 0.804 | 0.051 | 93.7 |
| 3.0 | 1.80 | 0.146 | 91.9 |
| 10.0 | 5.44 | 0.346 | 93.6 |
| 30.0 | 8.99 | 0.706 | 92.2 |
| 100.0 | 9.98 | 1.02 | 89.8 |

Uptake is expressed as mean saturable influx velocity over a 30-min incubation period after subtraction of non-saturable uptake; comparisons at each substrate concentration are made between paired contralateral ganglia from the same rat.

have no direct agonist activity (N.G. Bowery, personal communication).

(3) The concentration-sensitivity of low-Na$^+$ effects (Figure 3) follows from the saturable nature of carrier-mediated transport, such that proportionately less GABA is cleared from the interstitial spaces as the apparent Michaelis constant ($K_T \sim 7\ \mu$M; Bowery & Brown, 1972) is approached or exceeded (the fractional inhibition of uptake in low Na$^+$ being independent of substrate concentration).

Further consideration of the lateral shift in the dose-response curve in Figure 3 suggests that, at a nominal external GABA concentration of 10 µM in normal (143 mM [Na$^+$]) solution the effective interstitial concentration is only around 3 µM, i.e., the steady-state concentration in the tissue is reduced some 70% by the glial transport process. At a nominal external concentration of 3 µM, a larger reduction to 0.6 µM interstitial concentration appears to result. One consequence of this is that the previously-obtained potency measurements, made in normal Na$^+$ solution with a functional carrier (Bowery & Brown, 1974) need revision. In particular, if corrected for the influence of the carrier, GABA becomes equipotent with 3-APS as an agonist, instead of one-third as potent as previously deduced (the potency measurements having been made from the apparent $ED_{50}$ values).

Thus, the present observations imply that neuroglial cells in sympathetic ganglia are capable of net inward GABA transport at a rate sufficient to reduce the interstitial concentration of GABA. This may have some functional significance, since the concentration of GABA in the plasma has been estimated at 2–4 µM in cats (Crowshaw, Jessup & Ramwell, 1967). Inspection of Figure 3 shows that, in the absence of carrier-transport, such a concentration may produce an appreciable depolarization of the ganglion cells, but that carrier-mediated transport can reduce this effect to negligible levels. This would accord with inferences to be drawn from measurements of labelled GABA influx, which appears to drive towards a steady-state intracellular/extracellular concentration gradient of around $10^3$. At a resting intracellular concentration of some 0.5–1 mM (cf. Bowery *et al.*, 1976; Bertilsson, Suria & Costa, 1976), the glial cells would tend to 'buffer' interstitial GABA to about 1 µM, sufficient to preclude untoward effects on neuronal excitability (cf. Adams & Brown, 1975). The same considerations might well apply to other peripheral neurones sensitive to GABA, such as autonomic and somatic sensory neurones (De Groat, 1972; De Groat, Lalley & Saum, 1972), which are also invested by GABA-transporting neuroglial cells (Bowery & Brown, 1972; Young *et al.*, 1973; Schon & Keily, 1974).

Recently, Curtis, Game & Lodge (1976) have found that inhibitors of GABA transport in the brain (nipecotic acid, L-2,4-diaminobutyric acid and 2,2-dimethyl-β-alanine) applied by iontophoresis to a variety of central neurones potentiated the inhibitory effect of iontophoretically-applied GABA, thus indicating a role for membrane transport in reducing extracellular GABA levels in the central nervous system. At this site clearance may be affected through transport into either neurones or glial cells or both. As the authors point out, their experiments did not clearly indicate the relative importance of neural and glial transport processes, but the effect of the β-alanine derivative suggests at least a partial role of glial transport (cf. Johnston & Stephanson, 1976). The present experiments, on a tissue without a neural carrier, support this inference.

This investigation was aided by grants from the Medical Research Council to D.A. Brown and from the Peel Medical Research Trust to J.P. Quilliam and D.A. Brown. M. Galvan is in receipt of an M.R.C. training award. We thank Mr C. Cooksey of the Department of Chemistry, University College London for synthesizing (±)-nipecotic acid.

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*(Received October 27, 1976.)*

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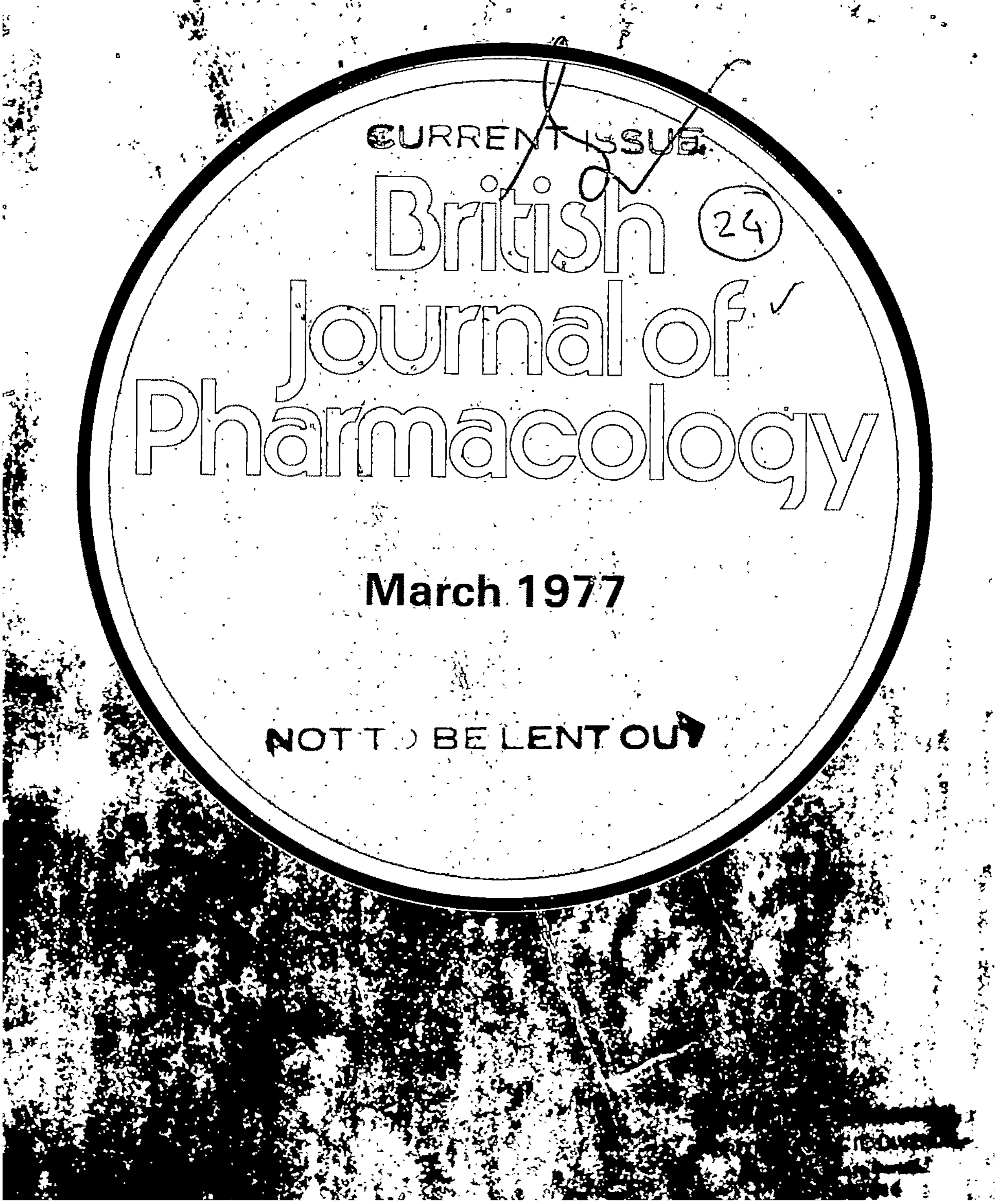
Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge

Volume 59(3)
ISSN 0007-1188

pages 379–434
435P–535P



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526P RICHARDS, C.D. & TEGG, W.J.B. A superfusion chamber suitable for maintaining mammalian brain tissue slices for electrical recording

527P HORSFALL, G.B., OLDHAM, A.A. & ROUSE, W. A novel and inexpensive method for detecting and quantitating the occurrence of cardiac arrhythmias

527P CUELLO, A.C. & KELLY, J.S. Electron microscopic autoradiographic localization of [$^3$H]-dopamine in the dendrites of the dopaminergic neurones of the rat substantia nigra *in vivo*

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*Br. J. Pharmac.* (1977), **59**, 385–391

# PROSTAGLANDIN $F_{2\alpha}$ REDUCES THE ALGESIC EFFECT OF BRADYKININ BY ANTAGONIZING THE PAIN ENHANCING ACTION OF ENDOGENOUSLY RELEASED PROSTAGLANDIN E

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1 The isolated perfused ear of the rabbit connected to the body only by its nerve, was used to investigate the influence of prostaglandin $F_{2\alpha}$ on the algesic effect of bradykinin and acetylcholine.

2 Bradykinin and acetylcholine, following intra-arterial injection into the isolated perfused ear elicited a dose-related reflex fall in blood pressure due to stimulation of paravascular pain receptors (= algesic effect).

3 Infusion of prostaglandin $F_{2\alpha}$ (0.1 to 1 ng/ml) into the rabbit ear reduced the algesic effect of bradykinin but not that of acetylcholine.

4 The onset of the reflex fall in blood pressure by bradykinin but not that by acetylcholine was delayed by infusion of prostaglandin $F_{2\alpha}$ into the ear.

5 Infusion of prostaglandin $E_1$ into the rabbit ear led to an enhancement of the algesic effect of bradykinin and acetylcholine. Enhancement of both effects was abolished by infusion of prostaglandin $F_{2\alpha}$.

6 During inhibition of the endogenous synthesis of prostaglandins (mainly E-type) by indomethacin, a low concentration of prostaglandin $F_{2\alpha}$ no longer reduced the algesic effect of bradykinin. However, a high concentration of $F_{2\alpha}$ continued to enhance the effect of bradykinin and acetylcholine.

7 Prostaglandin $F_{2\alpha}$ influenced neither the brief reduction in venous outflow produced by bradykinin nor the brief increase in venous outflow caused by acetylcholine.

8 The results suggest that prostaglandin $F_{2\alpha}$ does not directly reduce the effect of bradykinin but inhibits the enhancement of its algesic effect produced by prostaglandin E that is released endogenously by bradykinin. That the algesic effect of acetylcholine is not reduced by prostaglandin $F_{2\alpha}$ is in keeping with its releasing very little endogenous prostaglandin E.

## Introduction

Prostaglandins of the E and F series exert opposite effects in a variety of situations but so far nothing has been reported of opposing effects on 'pain receptors', represented by sensory nerve endings. However, there are a few reports concerning a pro-inflammatory effect of E prostaglandins and an anti-inflammatory effect of F-prostaglandins: prostaglandin $E_1$, given intradermally in rats, potentiated the increase in vascular permeability induced by bradykinin while prostaglandin $F_{2\alpha}$ inhibited the response (Willoughby, 1968; Thomas & West, 1973; 1974). Furthermore, the increased vascular permeability caused by intradermal prostaglandins $E_1$ and $E_2$ in rats was reduced by prostaglandin $F_{2\alpha}$ (Crunkhorn & Willis, 1969; 1971).

In earlier experiments on vascular pain receptors we used the rabbit isolated perfused ear méthod of Mirzojan & Doblatian (1955) and Mietkiewski (1956). We found that intra-arterial injection of algesic substances into the isolated perfused ear of the rabbit stimulated paravascular pain receptors, elicited action potentials in the great auricular nerve and produced a reflex fall in arterial blood pressure. Bradykinin and acetylcholine were found to be the most effective substances (Juan & Lembeck, 1973; 1974b). Prostaglandin $E_1$ strongly enhanced the algesic effect of all substances used and most that of bradykinin (Juan & Lembeck, 1974b). Prostaglandin $E_2$ was less active (Lembeck & Juan, 1974). Our findings with bradykinin and E prostaglandins confirmed the results of other authors (Ferreira, Moncada & Vane, 1973) on other preparations (see Discussion section).

In this paper we describe the opposing effects of E and F prostaglandins on the algesic effect of bradykinin and acetylcholine.

## Methods

### *Rabbit ear preparation separated from the systemic circulation*

Details of the method have been described earlier (Juan & Lembeck, 1974b). Rabbits of either sex (2.3–3.2 kg) were anaesthetized with pentobarbitone (35 mg/kg). An ear was perfused via its artery; the flow rate was adjusted by pressure to 5 ml/min (Tyrode solution bubbled with 95% $O_2$ and 5% $CO_2$, 37°C). The ear was left connected to the body by the great auricular nerve only. Injections of bradykinin and acetylcholine into the arterial inflow of the ear elicited a reflex fall in systemic arterial pressure of the lightly anaesthetized animal by stimulation of paravascular pain receptors. The fall in blood pressure, which in contrast to the rise in blood pressure in the dog after knee-joint stimulation (Ferreira, Moncada & Vane, 1973) was dose-related, was used as a quantitative parameter for the algesic effect. Blood pressure was measured in a carotid artery by a Statham pressure transducer P 23 D b and monitored with a Beckman type R 411 dynograph. Some ear preparations did not respond to bradykinin or became insensitive during the first 30 to 60 min of the experiment; these were discarded.

The venous outflow was measured with an electronic drop recorder connected to a Beckman type R 411 dynograph and changes were expressed as percentages of initial values.

### *Outline of the experimental procedure*

Bradykinin and acetylcholine were injected into the arterial inflow cannula of the ear in 2 or 3 different quantities at the beginning of the experiments to obtain the control values. Thereafter, prostaglandin $F_{2\alpha}$ was infused into the ear in three concentrations (0.1, 1.0 and 10.0 ng/ml) for more than 60 minutes. The injections of bradykinin and acetylcholine were repeated during the infusion at constant intervals.

In another series of experiments bradykinin and acetylcholine were injected in two different amounts (bradykinin: 30 ng and 100 ng; acetylcholine: 3 µg and 10 µg). The lower amounts of each produced trivial reflex falls in blood pressure. Then infusion of 10.0 ng/ml prostaglandin $E_1$ was started and maintained during the whole experiment. Prostaglandin $E_1$ enhanced the algesic effect of bradykinin and acetylcholine as reported earlier (Juan & Lembeck, 1974b). About 30 min after the start of the infusion the enhancement of the effects of bradykinin and acetylcholine was almost maximal. These enhanced effects ($C_E$ in Figure 3) served now as controls for the responses following additional infusions of prostaglandin $F_{2\alpha}$. Thirty-five minutes after the start of the infusion of prostaglandin $E_1$ an infusion of 50 ng/ml prostaglandin $F_{2\alpha}$ was given in addition to that of prostaglandin $E_1$ and maintained for 65 min (Figure 3). The injections of bradykinin and acetylcholine were repeated during the influence of prostaglandin $F_{2\alpha}$.

In further experiments prostaglandin $F_{2\alpha}$ and $E_1$ were injected in single doses 2 min before the injection of bradykinin or acetylcholine to obtain the control values. The injections were repeated during infusion with indomethacin 1 µg/ml for at least 30 minutes.

Statistical significance between means (against controls) was assessed by the paired *t* test.

### *Drugs*

The following drugs were used: acetylcholine chloride (Becker, Vienna); synthetic bradykinin (Sandoz, Basel) and prostaglandins $E_1$ and $F_{2\alpha}$ (Upjohn Co., Kalamazoo). All drug concentrations are quoted in terms of the base or acid.

## Results

### *Influence of prostaglandin $F_{2\alpha}$ on the algesic effect of bradykinin and acetylcholine*

During infusion of 0.1 ng/ml prostaglandin $F_{2\alpha}$ (Figure 1) the algesic effect of bradykinin was reduced significantly ($P < 0.01$) and 1.0 ng/ml prostaglandin $F_{2\alpha}$ caused even greater reduction (Figure 2). During infusion of the high concentration of 10.0 ng/ml prostaglandin $F_{2\alpha}$, however, the algesic effect of bradykinin was hardly reduced at all. After the end of the infusion of prostaglandin $F_{2\alpha}$, the effect of bradykinin was restored slowly, taking more than 100 minutes.

In contrast the effect of acetylcholine was never reduced by prostaglandin $F_{2\alpha}$ in any of the three concentrations used (Figures 1 and 2).

### *Interaction between prostaglandin $E_1$ and $F_{2\alpha}$*

During the concomitant infusion of prostaglandin $E_1$ (10 ng/ml) and prostaglandin $F_{2\alpha}$ (50 ng/ml) into the ear (Figure 3), the prostaglandin $E_1$-enhanced effects of bradykinin and of acetylcholine were reduced significantly ($P < 0.01$). After terminating the infusion of prostaglandin $F_{2\alpha}$, prostaglandin $E_1$ was infused for a further period. Responses to both bradykinin and acetylcholine resumed their previous magnitude ($C_E$ Figure 3) during a recovery period of 80 to 120 min for bradykinin and about 60 min for acetylcholine.

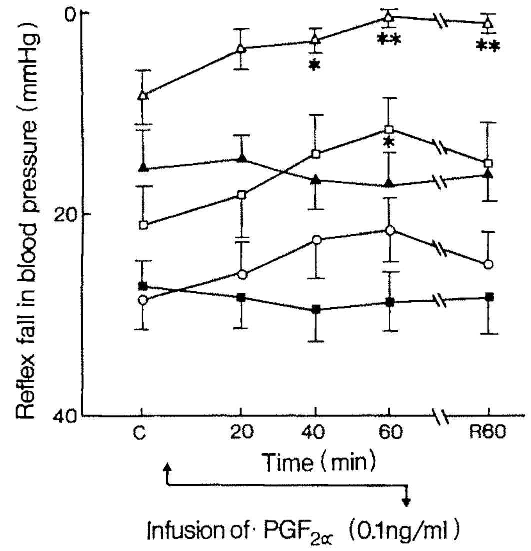

**Figure 1** Rabbit ear separated from the systemic circulation. Influence of infusion of 0.1 ng/ml prostaglandin $F_{2\alpha}$ on reflex fall in mean arterial blood pressure produced by bradykinin: 0.1 μg (△), 0.3 μg (□) and 1.0 μg (○) and acetylcholine: 10 μg (▲) and 30 μg (■). Controls=C. R60=recovery, 60 min after the end of infusion. Each point represents the mean of results from 6 rabbits. Vertical bars represent s.e. mean. $^*P<0.05$; $^{**}P<0.01$.

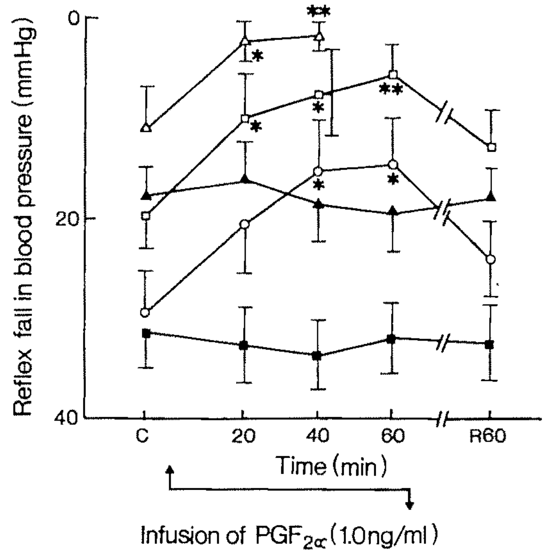

**Figure 2** Rabbit ear separated from the systemic circulation. Influence of infusion of 1.0 ng/ml prostaglandin $F_{2\alpha}$ on reflex fall in mean arterial blood pressure produced by bradykinin: 0.1 μg (△), 0.3 μg (□) and 1.0 μg (○) and acetylcholine: 10 μg (▲) and 30 μg (■). Controls=C. R60=recovery, 60 min after the end of infusion. Each point represents the mean of results from 5 rabbits. Vertical bars represent s.e. mean. $^*P<0.05$; $^{**}P<0.01$.

### *Influence of prostaglandin $E_1$ and $F_{2\alpha}$ on the delay time of the pain reflex response*

The delay time of the pain reflex response (time between injection and onset of reflex fall in blood pressure) is longer for bradykinin than for acetylcholine (Juan & Lembeck, 1974b).

During infusion of prostaglandin $F_{2\alpha}$, the delay of the response to acetylcholine remained unchanged while that of the response to bradykinin increased significantly ($P<0.01$) (Figure 4).

In another series of experiments the delays of the response to acetylcholine and bradykinin were unchanged by infusion of 10 ng/ml prostaglandin $E_1$. However, when in addition to prostaglandin $E_1$, prostaglandin $F_{2\alpha}$ (50 ng/ml) was also infused into the ear, the delay of the response to bradykinin was again significantly increased while that of the response to acetylcholine was not changed significantly (Figure 4).

### *Influence of prostaglandin $E_1$ and $F_{2\alpha}$ on the vasoactivity of bradykinin and acetylcholine*

When bradykinin was injected intra-arterially into the rabbit ear, the venous outflow showed a shortlasting decrease. Following injection of small amounts of acetylcholine (10 μg), the venous outflow slightly increased.

In our experiments the perfusion rate was kept constant by inflow pressure adjustment so that a possible vasoconstriction produced by infusion of prostaglandin $F_{2\alpha}$ may have been compensated for without recognition. Therefore, we did not measure the influence of prostaglandin $F_{2\alpha}$ on venous outflow but only its influence on the shortlasting response to injected bradykinin and acetylcholine. The vasodilator effect of 10 μg acetylcholine was slightly increased during infusion of prostaglandin $E_1$ and $F_{2\alpha}$, separately or together. The vasoconstrictor effect of bradykinin was not altered during infusion of prostaglandin $F_{2\alpha}$, was reduced significantly ($P<0.05$) during infusion of prostaglandin $E_1$ and was slightly reduced during the infusion of both prostaglandins together.

### *Influence of prostaglandin $F_{2\alpha}$ on the algesic effect of bradykinin and acetylcholine in the presence of indomethacin*

A single injection of 0.1 μg prostaglandin $F_{2\alpha}$ 2 min before the injection of bradykinin (0.3 μg) significantly reduced the algesic effect of bradykinin (Figure 5). The effect of acetylcholine (10 μg) was again not influenced. During infusion of indomethacin 1 μg/ml the effect of bradykinin became attenuated so that larger amounts were required to produce the same

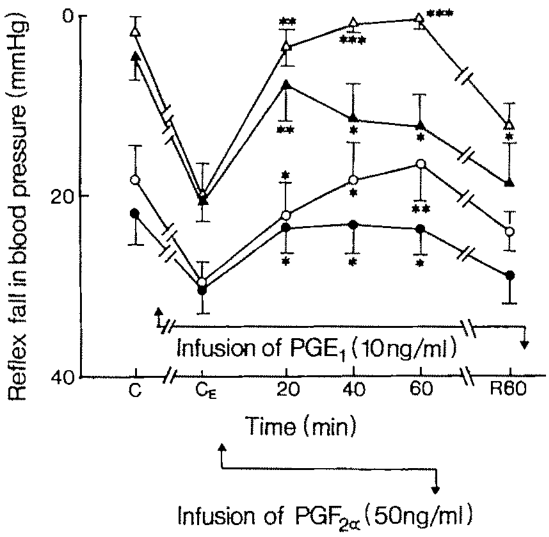

**Figure 3** Rabbit ear separated from the systemic circulation. Reflex fall in mean arterial blood pressure elicited by bradykinin: 0.03 µg (△), and 0.1 µg (○) and acetylcholine: 3 µg (▲) and 10 µg (●). C=controls. $C_E$='Controls', 30 min after start of infusion of 10 ng/ml prostaglandin $E_1$,-enhanced effect of bradykinin and acetylcholine. R60=recovery, 60 min after the end of infusion of prostaglandin $F_{2\alpha}$. Each point represents the mean of results from 6 rabbits. Vertical bars represent s.e. mean. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$ (against $C_E$).

response (3 µg instead of 0.3 µg). Under these conditions 0.1 µg prostaglandin $F_{2\alpha}$ no longer reduced the bradykinin response. However, a higher dose (10 µg) significantly ($P<0.01$) enhanced the effect of bradykinin (Figure 5). The effect of acetylcholine was also not influenced by 0.1 µg prostaglandin $F_{2\alpha}$ but was significantly ($P<0.01$) enhanced by 10 µg. Prostaglandin $E_1$ (0.1 µg) also significantly enhanced the response to bradykinin (Figure 5) and to acetylcholine during indomethacin infusion.

## Discussion

Earlier findings showed that E prostaglandins enhanced the algesic effect of bradykinin by a sensitizing action on pain receptors. Such a sensitizing action of E prostaglandins has been found in the human skin (Ferreira, 1972), the rat paw (Willis & Cornelsen, 1973), the spleen (Ferreira, Moncada & Vane, 1973), the knee joint (Ferreira, Moncada & Vane, 1974b; Moncada, Ferreira & Vane, 1974; 1975) and the heart (Vane, 1976) of dogs, and the ear of rabbits (Lembeck & Juan, 1974; Juan & Lembeck, 1974a; 1974b; 1976a; 1976b). Juan & Lembeck (1974b) found on the isolated ear preparation of the rabbit that E prostaglandins enhanced the effect of each of several substances which produced pain following intra-arterial injection, of which the most active were bradykinin and acetylcholine. The effects of these two substances on the rabbit ear preparation differ in the following respects: (a) The algesic effect of bradykinin is strongly attenuated by indomethacin whereas that of acetylcholine is only slightly reduced (Lembeck & Juan, 1974). (b) The prostaglandin antagonist, polyphloretin phosphate, inhibits the algesic effect of bradykin but not that of acetylcholine (Juan & Lembeck, 1976b). (c) Bradykinin in doses eliciting a pain reflex, released some nanograms of E prostaglandins, whereas acetylcholine released barely detectable amounts (Juan & Lembeck, 1976a and c; Lembeck, Popper & Juan, 1976).

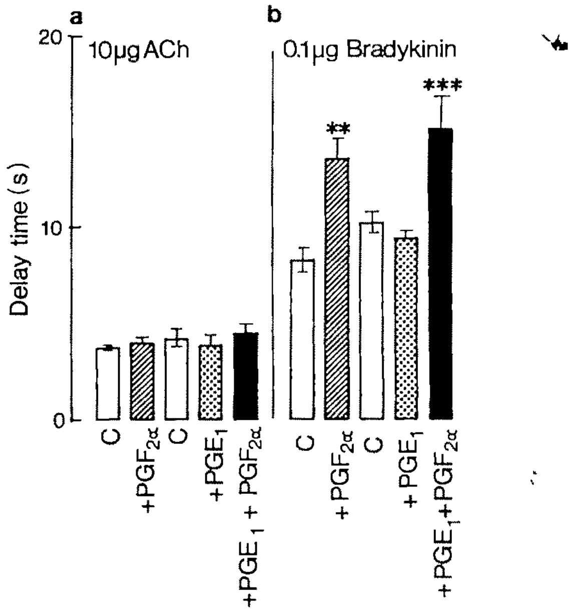

**Figure 4** Rabbit ear separated from the systemic circulation. Delay time of reflex fall in blood pressure elited by (a) 10 µg acetylcholine (ACh) and (b) 0.1 µg bradykinin: open columns=controls (C); cross-hatched columns=after infusion of 10 ng/ml prostaglandin $F_{2\alpha}$ for 30–50 min; dotted columns=after infusion of 10 ng/ml prostaglandin $E_1$ for 20–30 min; solid columns=after infusion of 50 ng/ml prostaglandin $F_{2\alpha}$ concomitantly with 10 ng/ml prostaglandin $E_1$ for 30–50 min. Each column represents the mean of results from 6 rabbits. Vertical bars show s.e. mean. $^{**}P<0.01$; $^{***}P<0.005$.

Prostaglandin of the E type is also released by bradykinin from the kidney (McGiff, Terragno, Malik & Lonigro, 1972) and spleen (Ferreira, Moncada & Vane, 1973) of dogs, the lung of guinea-pigs (Palmer, Piper & Vane, 1973) and the heart of rabbits

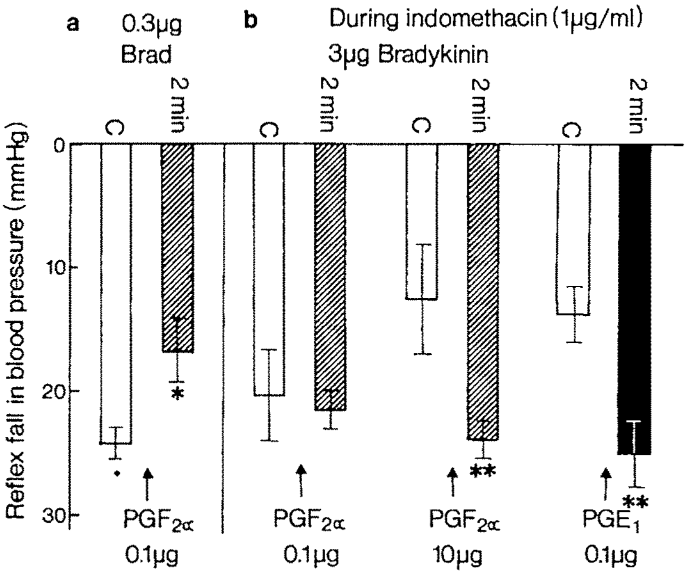

**Figure 5** Rabbit ear separated from the systemic circulation. Effect of injection of 0.1 or 10 μg of prostaglandin $F_{2\alpha}$ (cross-hatched columns) and 0.1 μg prostaglandin $E_1$ (solid column) on reflex fall in mean arterial blood pressure produced by bradykinin (a) 0.3 μg or (b) 3 μg before and more than 30 min after start of infusion of 1 μg/ml indomethacin. C (open columns) = controls; 2 min = bradykinin response 2 min after the prostaglandin. Each column represents the mean of results from 7 rabbits. Vertical bars show s.e. mean. $^*P<0.05$; $^{**}P<0.01$.

(Needleman, Marshall & Sobel, 1975). The released E prostaglandins sensitize the pain receptors to the action of bradykinin. Accordingly, indomethacin reduces but does not abolish the algesic effect of bradykinin in dog spleen (Ferreira, Moncada & Vane, 1973) and in the rabbit ear preparation (Juan & Lembeck, 1974a; Lembeck & Juan, 1974) by inhibition of prostaglandin release or synthesis (Ferreira, Moncada & Vane, 1971; Smith & Willis, 1971; Vane, 1971). Polyphloretin phosphate also reduces the effect of bradykinin in the rabbit ear but by interference with the pain enhancing effect of the endogenously released E prostaglandins (Juan & Lembeck, 1976b). Prostaglandin $F_{2\alpha}$, in doses 20 times the dose of prostaglandin $E_1$, induced incapacitation in the dog knee joint (Rosenthale, Dervinis, Kassarich & Singer, 1972) but did not sensitize pain receptors to bradykinin (Moncada, Ferreira & Vane, 1975). Again, in contrast to prostaglandin $E_1$, prostaglandin $F_{2\alpha}$ did not elicit abdominal contractions on intraperitoneal injection into mice, indicating lack of nociceptive effect (Collier & Schneider, 1972).

In this study prostaglandin $F_{2\alpha}$ infused intra-arterially into the rabbit isolated perfused ear in very low concentrations (0.1–1.0 ng/ml) reduced the algesic effect of bradykinin but not that of acetylcholine. Surprisingly, a high concentration (10.0 ng/ml) did not further reduce the effect of bradykinin; only slight reductions or slight enhancements were observed. Perhaps prostaglandin $F_{2\alpha}$ in this high concentration has some intrinsic agonist activity on pain receptors. In contrast the effect of acetylcholine was not reduced by any concentration of prostaglandin $F_{2\alpha}$. From these results it could be deduced that the exogenously applied prostaglandin $F_{2\alpha}$ reduced the algesic effect of bradykinin by inhibiting the pain enhancing action of the endogenously released E prostaglandins. In keeping with this, an infusion of prostaglandin $F_{2\alpha}$ reduced the enhancement by prostaglandin $E_1$ of the algesic effect of both bradykinin and acetylcholine.

The experiments with indomethacin confirm this assumption. During infusion of 1 μg/ml indomethacin the release of prostaglandins by bradykinin from the rabbit ear is abolished (Lembeck, Popper & Juan, 1976). Under these conditions a low dose of prostaglandin $F_{2\alpha}$ no longer reduced the bradykinin effect. However, a high dose of prostaglandin $F_{2\alpha}$ enhanced the effect of bradykinin, clearly showing that it has some intrinsic activity which in Figure 5, is seen to be about one hundredth that of prostaglandin $E_1$.

The results with low concentrations of prostaglandin $F_{2\alpha}$ in the rabbit ear are similar to those obtained by other authors in other systems. Thus, Brody & Kadowitz (1974) found that prostaglandin $F_{2\alpha}$, which exerted no direct effect on vascular resistance in the perfused dog uterus, markedly attenuated the vasodilator response to prostaglandin $E_1$. Both prostaglandins are thought to compete for the same receptors in this system as was also supposed to be so in the uterine artery of dogs (Clark & Brody, 1974) in stomach strips (Splawinski, Nies, Sweetman & Oates, 1973) and in the mouse ovary (Kuehl, Cirillo, Ham & Humes, 1973). A similar interaction seems to exist on or near adrenergic nerve terminals; when neurogenically evoked vasoconstrictor responses were depressed by infusion of prostaglandin $E_1$, infusion of prostaglandin $F_{2\alpha}$ specifically restored the response to nerve stimulation (Kadowitz, Sweet & Brody, 1971).

Some additional observations on the delay time of the reflex response and the venous outflow were made. These indicate that the different effects of prostaglandins on pain receptors are not secondary to vascular actions. Prostaglandin $F_{2\alpha}$ prolonged the delay time of the reflex response to bradykinin but not to acetylcholine, independent of a simultaneous infusion of prostaglandin $E_1$. The reason for this prolongation of delay remains unknown. A vascular action which causes a delay of the access of bradykinin to the pain receptors appears to be rather unlikely since prostaglandin $F_{2\alpha}$ did not change the vasoconstrictor effect of bradykinin; it reduced only moderately the vasoconstrictor effect of bradykinin attenuated by prostaglandin $E_1$. The brief vasodilator effect of acetylcholine was slightly greater in the presence of both prostaglandins than under control conditions.

Prostaglandins have been identified in inflammatory exudates of various origins and have been assumed to play a role in inflammation (see Ferreira, Moncada & Vane, 1974a). The ratio of E and F prostaglandins (E/F) seems to be important for the inflammatory response (Giroud, Velo, Timsit & Willoughby, 1974). We found that prostaglandins of the F series are released in much smaller amounts by bradykinin than are E prostaglandins (Juan & Lembeck, 1976c; Lembeck, Popper & Juan, 1976). But concentrations of as low as 0.1 ng/ml prostaglandin $F_{2\alpha}$ were sufficient to antagonize the pain enhancing action of prostaglandin $E_1$. Similar concentrations of prostaglandin $F_{2\alpha}$ specifically increased responses to sympathetic nerve stimulation and increased the amount of catecholamines released from the adrenal gland by splanchnic nerve stimulation (Brody & Kadowitz, 1974).

Thus, small amounts of F prostaglandins released by bradykinin or by other stimuli under *in vivo* conditions could occupy prostaglandin binding sites on or near paravascular 'pain receptors' and could antagonize the pain enhancing action of released (or applied) E prostaglandins. In this way interactions of E and F prostaglandins could regulate or modulate algesic actions at the receptor site, the ratio of their concentrations being considered an important factor.

This investigation was supported by grant No. 2575 from the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich. The authors wish to thank Mr W. Sametz for his skilful technical assistance. We also thank Dr J. Pike for the gift of prostaglandins and Dr E. Stürmer for providing synthetic bradykinin.

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*(Received July 1, 1976.*
*Revised October 5, 1976.)*

*Br. J. Pharmac.* (1977), 59, 393–395

# BRAIN AND VASCULAR MONOAMINE OXIDASE ACTIVITY IN THE DEOXYCORTICOSTERONE-SALT HYPERTENSIVE RAT

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1 The role of monoamine oxidase (MAO) in the maintenance of deoxycorticosterone-sodium chloride (DOCA-salt) hypertension was investigated by assaying the MAO activity both in central as well as peripheral blood vessels and in brain tissue.

2 The results suggest that the activity of MAO in the DOCA-salt hypertensive rat is similar to the activity present in the normotensive rat.

## Introduction

Many workers have reported on the importance of the central nervous system in the development or maintenance of various forms of experimental hypertension, such as renal hypertension (Chalmers, Dollery, Lewis & Reid, 1974), deoxycorticosterone (DOCA)-salt hypertension (Finch, Haeusler & Thoenen, 1972), and hypertension induced by lesions of the anterior hypothalamus in the rat (Nathan & Reis, 1975). It has also been suggested (de Champlain, Mueller & Axelrod, 1969; Reid, Zivin & Kopin, 1975) that DOCA-salt hypertension causes increased peripheral adrenergic activity through a central component.

In DOCA-salt hypertension, there is an increased urinary excretion of noradrenaline (NA) and its metabolites (de Champlain *et al.*, 1969) and, in addition, the cardiac NA turnover rate is increased (de Champlain *et al.*, 1969, Nakamura, Gerold & Thoenen, 1970). Though the half-life of cardiac NA was short and more NA and its metabolites were present in the urine of DOCA-salt hypertension, cardiac catechol-*O*-methyl tranferase enzyme in DOCA-salt hypertensive rats was found to be similar to that in the controls (de Champlain, Krakoff & Axelrod, 1968). Although cardiac monoamine oxidase (MAO) was increased in the hypertensive rats, the elevation was due to the cardiac hypertrophy which occurred during the DOCA-salt treatment (de Champlain *et al.*, 1968).

Recently Reid *et al.* (1975) have shown that although the plasma NA is increased in the DOCA-salt induced hypertensive rat, there is no parallel increase in the plasma dopamine-$\beta$-hydroxylase. Since an appreciable fraction of circulating NA and NA metabolites appearing in urine may be derived from the sympathetic supply to blood vessels (Bigelow, Dairman, Weil-Malherbe & Udenfriend, 1968; Berkowitz, Tarver & Spector, 1971), and limited information on vascular MAO in DOCA-salt hypertension is available, we decided to examine the role of vascular MAO in the maintenance of DOCA-salt hypertension.

## Methods

Uninephrectomized male Wistar rats (8 weeks old) obtained from Charles River Breeding Lab. Inc., Wilmington, Mass., were divided into two groups, each fed a regular laboratory diet. One group of rats was made hypertensive by the subcutaneous injection of a suspension of DOCA acetate (ICN Pharmaceuticals, Inc., Cleveland, Ohio), 5 mg/kg twice a week for a period of seven weeks, and the addition of 1% NaCl solution in their drinking water. The other group of uninephrectomized rats were allowed free access to the drinking water containing 1% NaCl but were not treated with DOCA acetate and thus served as the control group. The systolic blood pressure of the rats in both groups was measured by a tail cuff microphone method using an instrument made by Hoffmann-LaRoche & Co. Ltd., Basle, Switzerland.

Animals were killed by decapitation and their brains removed. The brain microvessels were prepared by a modification of the method of Brendel, Meezan &

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Carlson (1974), as previously described (Lai, Udenfriend & Spector, 1975). The brain filtrate was designated as that fraction of the homogenate which passed through the nylon mesh. Vascular tissue, i.e. aorta, mesenteric artery and mesenteric vein, was removed and perivascular adipose tissue carefully dissected away from the vascular tissue.

MAO activity was determined by the method of Goridis & Neff (1971) with the modification that the substrate (tyramine) concentration was increased to 2.5 mM.

All tissues were homogenized in phosphate buffer (67 mM; pH 7.2). Brain microvessels (from four brains) were homogenized in 120 µl; mesenteric arteries and veins in 20 volumes, and aortae in 15 volumes. The reaction mixture consisted of 2.5 mM tyramine containing about $8.5 \times 10^4$ ct/min of [1-$^{14}$C]-tyramine plus the tissue homogenate, in a total volume of 140 µl of buffer solution. The mixture was incubated for 30 min at 37°C and the reaction was terminated by the addition 40 µl of 60% perchloric acid. After centrifugation of the sample, the supernatant was passed over a Rexyn 101 column ($H^+$, 200–400 mesh) and washed with 5 ml of distilled water. The effluent and washes were collected and mixed with 15 ml of Aquasol in a counting vial. Radioactivity was measured in a Beckman Liquid Scintillation Counter. Tissue protein was determined according to the method of Lowry, Rosebrough, Farr & Randall (1951).

## Results

After seven weeks of treatment with DOCA-salt, rats became hypertensive with systolic blood pressures (mean ± s.e., mmHg) (*n*) of 205.3 ± 5.6 (12), significantly ($P < 0.001$) higher than that of controls, 138 ± 3.6 (12). The DOCA-salt treated group also showed a reduced weight gain, final weights being 328.0 ± 12.2 g (12) compared to the control group 403.4 ± 8.2 g (12) ($P < 0.001$).

The central and peripheral vascular and brain MAO activities are summarized in Table 1. MAO activities in brain filtrate and brain microvessels of DOCA-salt hypertensive rats were not different from those of the controls. It is of great interest to note that the enzyme activity in brain microvessels in both DOCA-salt hypertensive and normotensive rats is 3.0-fold more than that of brain filtrate. The MAO activities in mesenteric arteries and veins and in aortae of hypertensive animals were found to be similar to those of the control but not as great as the activity found in brain microvessels. It also can be seen from the table that MAO activity in mesenteric vein is approximately two-fold that of mesenteric artery or aorta in both the hypertensive and control groups.

## Discussion

MAO is the enzyme responsible for the oxidative deamination of various biogenic amines found in many tissues. MAO also plays an important physiological role by controlling the level of intracellular monoamine at nerve terminals (Kopin, 1964). The presence of this degradative enzyme in blood vessels such as the mesenteric artery, mesenteric vein and aorta has been demonstrated elsewhere (Lai, Berkowitz & Spector, 1975). Our results show that the MAO activity in the vascular and brain tissue of the hypertensive animal is not different from the control. This would imply that the increased plasma NA observed during the development of DOCA-salt hypertension (Reid, *et al.*, 1975) is not due to the impairment of the MAO catalytic system in vascular tissues.

We have reported (Lai, Berkowitz & Spector, 1976) that following the inhibition of brain microvessels MAO by clorgyline, intraventricular injection of NA produces a profound pressor effect. We, therefore, proposed that the brain microvessel MAO might function to inactivate that NA which is synthesized in the central nervous system. It would thus prevent the NA from impinging on sites in the brain which might elicit hypertension. Additionally, it could perhaps prevent the efflux of NA to peripheral sites.

**Table 1** Monoamine oxidase (MAO) activity in various tissues of normotensive and DOCA-salt hypertensive rats

| | *MAO activity (nmol mg$^{-1}$ protein h$^{-1}$)* | |
|---|---|---|
| *Tissues* | *Normotensive* | *Hypertensive* |
| Brain filtrate | 133.4 ± 9.6 (3) | 130.2 ± 17.2 (3) |
| Brain microvessels | 408.8 ± 45.9 (3) | 399.4 ± 56.8 (3) |
| Mesenteric artery | 63.6 ± 5.6 (7) | 68.9 ± 5.3 (7) |
| Mesenteric vein | 130.6 ± 11.3 (6) | 115.9 ± 8.4 (6) |
| Aorta | 57.3 ± 3.4 (6) | 60.3 ± 3.1 (6) |

Blood vessels were pooled and homogenized in phosphate buffer (67 mM, pH 7.2) for each experiment. Brain microvessels, from four brains, were homogenized in 120 µl phosphate buffer, mesenteric artery and vein were homogenized in 20 volumes of the buffer while aorta was homogenized in 15 volumes of buffer. Brain filtrates were collected from four grains and centrifuged at 120 *g* for 10 min at 4°C. The brain precipitate was then resuspended and homogenized in 160 ml of phosphate buffer and an aliquot of that was used for MAO assay. Figures represent mean ± s.e. Numbers in parentheses refer to the number of experiments.

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*(Received July 27, 1976.*
*Revised September 27, 1976.)*

*Br. J. Pharmac.* (1977), 59, 397–402

# THE SPECIFICITY OF OESTROGEN RECEPTOR IN BRAIN, PITUITARY AND UTERUS

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1 The specificity of oestrogen receptor in rat brain regions (hypothalamus, amygdala and cortex), pituitary and uterus was studied by measurement of the inhibition of 17$\beta$-oestradiol high affinity binding in cytosol, in the presence of unlabelled putative inhibitors of binding.

2 Binding was inhibited only by those of the compounds tested that possessed oestrogen agonist or antagonist activity. The affinities were estimated and the ranking order of the compounds was the same in all tissue sources of cytosol and corresponded to the ranking order of agonist or antagonist potency.

3 There were some significant differences between some of the estimated affinities in different tissues, these being seen most commonly between pituitary and hypothalamus on one hand and uterus on the other.

4 The possibility of heterogeneity of oestrogen receptor is discussed.

## Introduction

It is believed that many significant actions of steroid hormones are mediated by the formation of complexes with receptors in cytoplasm; the complexes so formed move to the nucleus where they bind to chromatin; this leads to a modification of genetic expression and thence to the physiological responses which are characteristic of the hormone on that particular tissue. As well as influencing secondary sexual organs, oestrogens affect brain and pituitary, to modify sex-linked behaviour and to control gonadotrophin secretion and cytosols from hypothalamus and pituitary. have been shown to contain high affinity receptors for oestrogens (Eisenfeld, 1970; Kahwanago, Heinrichs & Herrman, 1970; Notides, 1970; Vertes & King, 1971; Ginsburg, MacLusky, Morris & Thomas, 1971; 1972).

The specificity of those oestrogenic responses is therefore dependent on that of the initial hormone-receptor interaction. The specificity of the uterine receptor has been studied (Korenman, 1968; 1969; 1970; Notides, 1970; De Sombre, Chabaud, Puca & Jensen, 1971), but little is known about that of the reaction in the central nervous system and pituitary. We describe here the binding affinities of a number of oestrogens, antioestrogens and non-oestrogenic steroids for the cytosol receptors of cerebral cortex, amygdala, hypothalamus and pituitary of male and female rats and for uterine cytosol receptors. As it has been reported (Shani, Givant, Sulman, Eylath & Eckstein, 1971) that perphenazine inhibits the uptake of oestradiol into the hypothalamus we included it in our list of test substances.

## Methods

[2,4,6,7$^3$H]-oestradiol (85 Ci/mmol), obtained from the Radiochemical Centre, Amersham, England, was repurified and stored as described before (Ginsburg, Greenstein, MacLusky, Morris & Thomas, 1974).

Sources of unlabelled steroids and other putative inhibitors are shown in Table 1. They were stored in ethanol and diluted in buffer to the required concentration just before use.

### *Preparation of cytosol*

Phosphate buffer 0.01 M pH 7.4 containing 250 mM sucrose was used. Homogenization medium contained, in addition, 0.1 M 2-mercaptoethanol. Twelve week old female (selected at the metoestrous stage of the 4 day oestrous cycle) and male Wistar rats were beheaded and tissues (cerebral cortex, amygdala and hypothalamus, whole pituitary and uterus) were dissected into ice-cold homogenization medium and homogenized as described elsewhere (Ginsburg *et al.*, 1974). Homogenates were centrifuged (110,000 $r_{av} \times g \times 1$ h) to prepare cytosols.

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### *Measurement of binding*

[$^3$H]-oestradiol: equilibrium dissociation constants of reaction were determined as described in detail elsewhere (Ginsburg *et al.*, 1974). In summary, 0.2 ml aliquots of cytosols were incubated (30°C) with 0.1 ml aliquots of [$^3$H]-oestradiol ($10^{-9}$ M to $3 \times 10^{-10}$ M) in buffer for 10 min (hypothalamus), 20 min (pituitary) and 30 min (cortex and amygdala); times that are more than enough to allow equilibrium between the high affinity receptor and the hormone to be reached. On completion of incubation 0.2 ml aliquots of the incubation were applied to small columns of Sephadex LH20 (6 x 0.45 cm) and allowed to remain in contact with the gel at 0–4°C for 30–60 min, in which time much of the low affinity bound oestradiol is freed and fixed to the gel although the hormone-receptor complex remains intact. Some radioactivity from the low affinity high capacity complexes appears in the eluates; estimates of eluted oestradiol arising from this source were obtained from parallel incubation in the presence of excess diethylstilboestrol (DES). It is necessary to subtract these from total binding to obtain values for high affinity binding.

### *Measurement of the binding of unlabelled compounds in the high affinity oestradiol binding system*

Affinities were measured by competition with radioactive oestradiol. As well as incubating cytosols with [$^3$H]-oestradiol in the presence and in the absence of an excess of DES, we carried out parallel incubations in the presence of other competitors. Bound radioactivity was then separated from free in the usual way.

### *Calculation of affinities*

[$^3$H]-oestradiol: equilibrium dissociation constants of reaction ($K_d$), were calculated by the method of Scatchard (1949) who derived the equation $B/F = K_a(n - B)$ to describe the binding of a ligand in a solution of a macromolecule where $n$ is the concentration of equivalent and independent binding sites,

**Table 1** Unlabelled steroids and other putative inhibitors used

| *Trivial name (abbreviation)* | *Chemical name* | *Source* |
|---|---|---|
| *Steroids* | | |
| $^{3}$Progesterone | Δ 4 Pregnen-3, 20 dione | Sigma |
| $^{3}$Corticosterone | Δ 4 Pregnen-11$\beta$, 21 diol-3, 20 dione | Sigma |
| $^{3}$Testosterone | Δ 4 Androsten-17$\beta$ol, 3 one | Sigma |
| $^{3,4}$5$\alpha$-Dihydrotestosterone | 5$\alpha$ Androstan-17$\beta$ol, 3 one | Sigma |
| $^{5}$Cyproterone | 1,2$\alpha$ Methylene-6 chloro-4,6, pregnadien-17$\alpha$ol-3, 20 dione | *Schering Corp. |
| $^{4}$Androstan-3$\alpha$ diol (3$\alpha$ diol) | 5$\alpha$ Androstan-3$\alpha$, 17$\beta$ diol | Steraloids |
| $^{4}$Androstan-3$\beta$ diol (3$\beta$ diol) | 5$\alpha$ Androstan-3$\beta$, 17$\beta$ diol | Steraloids |
| $^{1,4}$17$\beta$-Oestradiol (17$\beta$E$_2$) | 1,3,5 (10) Oestratrien-3, 17$\beta$ diol | Sigma |
| $^{1}$17$\beta$-Oestradiol (17$\alpha$E$_2$) | 1,3,5 (10) Oestratrien-3, 17$\alpha$ diol | Sigma |
| $^{1}$Oestrone (E$_1$) | 1,3,5 (10) Oestratrien-3ol, 17one | Sigma |
| $^{1}$Oestriol (E$_3$) | 1,3,5 (10) Oestratrien-3, 16$\alpha$, 17$\beta$ triol | Steraloids |
| $^{1}$16-Epioestriol (Epi E$_3$) | 1,3,5 (10) Oestratrien-3, 16$\beta$, 17$\beta$ triol | Steraloids |
| $^{2}$Ru 2858 | 11$\beta$ Methoxy, 17$\alpha$ ethynyl-1,3,5 (10) oestratrien-3, 17$\beta$ diol | *Roussel |
| *Non-steroidal compounds* | | |
| $^{1}$Diethylstilboestrol (DES) | 3,4 Bis (4 hydroxyphenyl) Δ 3 hexane | Sigma |
| $^{2}$MER-25 | 1 (*p*-2 Diethylaminophenyl)-1 phenyl-2*p* methoxy phenyl ethanol | *Merrell |
| $^{2}$*cis*-Clomiphene (Cis Clo) | *cis*-Triethyl amino-2(*p*(2-chloro-1, 2 diphenylvinyl)phenoxy) monocitrate | *Merrell |
| $^{2}$*trans*-Clomiphene (T Clo) | *trans*-Triethyl amino-2(*p*(2-chloro-1,2 diphenylvinyl)phenoxy) monocitrate | *Merrell |
| Perphenazine | 2-Chloro-10 (3-(1-(2 hydroxyethyl)-4 piperazinyl) propyl) phenothiazine | *Schering |

* Compounds received as gifts either directly or through the late Dr P.G. MacDonald.

$^{1}$=oestrogens; $^{2}$=antioestrogens; $^{3}$= non-oestrogenic; $^{4}$= testosterone metabolites; $^{5}$=anti-androgens.

The distinction between oestrogens and antioestrogens, on the one hand, and 'non-oestrogens' on the other is of course not absolute. It is made here solely on the basis of primary biological effect. All of the 'non-oestrogens' used will, under certain circumstances, elicit oestrogen or anti-oestrogen-like responses.

where $B$ is the concentration of ligand/macromolecule complex and $F$ is the concentration of free ligand. $K_a = (1/K_d)$ is the equilibrium association constant of reaction. When the ratio $B/F$ is plotted against $B$ the points are fitted by a straight line of slope $-K_a$ and which intercepts the ordinate at $nK_a$ and the abscissa at $n$.

The affinities of the various competitors were calculated in two ways. Data from preliminary experiments to see whether the effects observed on oestradiol binding were competitive were analysed as follows:

The Edsall & Wyman (1958) equation states that for competitive interaction between a ligand and an inhibitor at a single class of binding sites

$$B = \frac{nK_aF}{1 + K_aF + K_iI}$$

where $I$ = the concentration of free inhibitor and $K_i$ is the equilibrium association constant of the receptor-inhibitor reaction.

By rearrangement, an expression similar in form to the Scatchard equation may be obtained:

$$\frac{B}{F} = \frac{K_a(n-B)}{1 + K_iI}$$

Comparing this with the Scatchard equation we may conclude that the sole effect of a fixed concentration of competitive binding inhibitor on a Scatchard plot of the binding of a ligand to a single class of non-interacting sites, will be to decrease the slope of the plot by a factor of $1/(1 + K_iI)$; neither the rectilinear nature of the plot, nor its intercept where $B/F = 0$, at the saturation binding capacity of the system, will be affected. In contrast, if binding inhibition is due, even in part, to non-competitive interaction then the term $K_a/(1 + K_iI)$ will vary with ligand concentration and the Scatchard plot, in the presence of the inhibitor, will be curvilinear.

At least one experiment was carried out with each inhibitor in which Scatchard plots were constructed from six values of $B$ and $F$ in both the presence and absence of fixed concentrations of inhibitors. For all the compounds that inhibited oestradiol binding, the Scatchard plot was shifted by a linear reflection about the intercept on the abscissa according to the prediction above for competitive inhibition. However, several replications of this procedure for each compound would have been prohibitive in terms of labour and material and our routine practice was to construct the Scatchard plot from 6 values of $B$ and $F$ in the absence of inhibitor and two values for $B$ and $F$ in the presence of a fixed concentration of each inhibitor.

$K_{di}$ (= $1/K_i$) was calculated from the following equation derived from the Edsall & Wyman equation:

$$K_{di} = \frac{1}{nK_a\dfrac{F}{B} - (K_aF + 1)}$$

thus $K_{di}$ can be obtained if $K_a$ and $n$ are known together with values for $F$ and $B$ in the presence of $I$.

## Results

In preliminary experiments, aliquots of cortex, amygdaloid region, hypothalamus, pituitary and uterus cytosols, from tissues of intact adult female rats were incubated with [$^3$H]-17$\beta$-oestradiol (final incubation concentration, $1 \times 10^{-9}$ M) either alone, or in the presence of a range of concentrations ($10^{-10}$ to $10^{-6}$M) of each of the unlabelled test compounds. Control incubates, in each case, contained the same concentrations of [$^3$H]-17$\beta$-oestradiol alone and with the various unlabelled compounds, in the presence of excess ($10^{-7}$ M) unlabelled DES. Following incubation, bound [$^3$H]-17$\beta$-oestradiol was separated from free steroid by Sephadex LH20 gel filtration, and high affinity binding assessed as previously described.

Results for each tissue cytosol studied were essentially similar: in all cases low affinity binding i.e. binding remaining in the presence of excess DES, was unaffected by the presence of any of the unlabelled compounds, even at the highest concentrations used. In contrast high affinity binding was inhibited, increasingly as the concentration was raised, of unlabelled 17$\beta$-oestradiol, diethylstilboestrol, 17$\alpha$-oestradiol, oestrone, oestriol, 16-epioestriol, Ru 2858, MER-25, *cis*-clomiphene, *trans*-clomiphene and androstan-3$\alpha$ diol. The remaining unlabelled compounds (corticosterone, progesterone, testosterone, 5$\alpha$-dihydrotestosterone, cyproterone and perphenazine) did not significantly inhibit high affinity binding of [$^3$H]-17$\beta$-oestradiol, in any cytosol fraction, at concentrations up to $10^{-6}$ M.

The nature of the interactions between [$^3$H]-17$\beta$-oestradiol, and the various unlabelled compounds found to inhibit high affinity cytosol binding was then studied further, as follows: from the results of the preliminary series of experiments, concentrations of each of the unlabelled inhibitors were chosen (Table 2) which produced only a partial inhibition of the high affinity binding of [$^3$H]-17$\beta$-oestradiol in cytosol incubate at a concentration $1 \times 10^{-9}$ M. Isotherms were then constructed for binding of [$^3$H]-17$\beta$-oestradiol to the high affinity sites in each of the tissue cytosols, in the presence and absence of these concentrations of the inhibitors.

Figure 1 shows Scatchard representations of the isotherms obtained with hypothalamic cytosol, in the presence and absence of unlabelled androstan-3$\alpha$,17$\beta$ diol ($3.3 \times 10^{-7}$ M). Both sets of data yield rectilinear Scatchard plots, intercepting the abscissa at the saturation binding capacity of the high affinity sites; however, in the presence of the inhibitor, the slope of the plot is markedly reduced. These results are consistent with a model for competitive interaction

**Table 2** Concentrations of unlabelled compounds found in preliminary experiments to inhibit high affinity binding of [$^3$H]-17$\beta$-oestradiol ($5 \times 10^{-10}$ M) in uterine cytosols, by between 30 and 70%

| | (M) |
|---|---|
| MER-25 | $3.3 \times 10^{-7}$ |
| Androstan-3$\alpha$ diol | $3.3 \times 10^{-7}$ |
| *trans*-Clomiphene | $3.3 \times 10^{-7}$ |
| Androstan-3$\beta$ diol | $3.3 \times 10^{-7}$ |
| *cis*-Clomiphene | $3.3 \times 10^{-8}$ |
| 17$\alpha$-Oestradiol | $3.3 \times 10^{-8}$ |
| Oestrone | $3.3 \times 10^{-8}$ |
| Oestriol | $3.3 \times 10^{-8}$ |
| 16 Epioestriol | $3.3 \times 10^{-8}$ |
| Ru 2858 | $3.3 \times 10^{-8}$ |
| 17$\beta$-Oestradiol | $1.3 \times 10^{-8}$ |
| Diethylstilboestrol | $6.7 \times 10^{-10}$ |

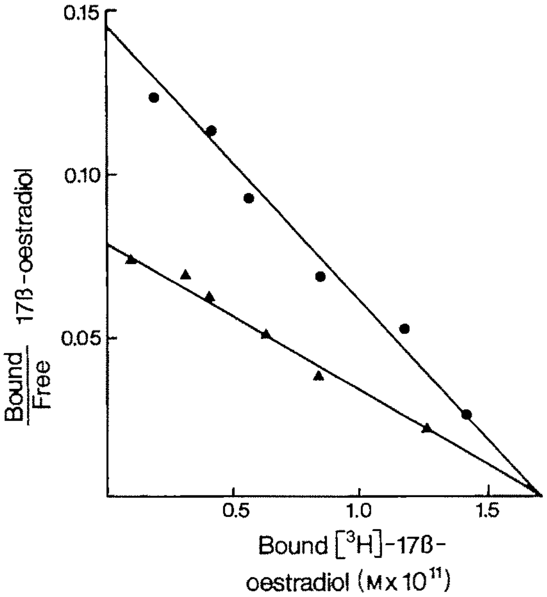

**Figure 1** Inhibition of [$^3$H]-17$\beta$-oestradiol binding in female rat hypothalamic cytosol by androstan-3$\alpha$ diol ($3.3 \times 10^{-7}$ M): (●) [$^3$H]-17$\beta$-oestradiol alone; (▲) [$^3$H]-17$\beta$-oestradiol plus androstan-3$\alpha$ diol.

between androstan-3$\alpha$,17$\beta$ diol and [$^3$H]-17$\beta$-oestradiol for the available binding sites.

Similar results were obtained with all other combinations found to inhibit [$^3$H]-17$\beta$-oestradiol binding with cytosols: from the 9 different classes of tissue source and with each of the 12 compounds, Scatchard representations of the binding isotherms were in all cases consistent with binding inhibition being due to competitive interaction between the inhibitors and [$^3$H]-17$\beta$-oestradiol at the high affinity sites. These experiments yielded values for $K_{di}$ and for each compound up to 4 additional estimates of the parameter were obtained by the more economical double point method.

The results were satisfactorily concordant with all tissues except cortex where the variability of the double point assays was unacceptably high. In view of the extremely low levels of high affinity binding present in cortex cytosols, this problem was ascribed to errors in the measurement of high affinity bound [$^3$H]-17$\beta$-oestradiol remaining in the presence of the inhibitors. The number of inhibitors studied in cortex cytosols was therefore restricted to 6 compounds (*cis*-clomiphene, 17$\alpha$-oestradiol, oestriol, oestrone, DES and unlabelled 17$\beta$-oestradiol) and each assay was carried out in duplicate so as to double the number

**Table 3** Molar dissociation constants of oestrogen agonists and antagonists in reaction with cytosol high affinity receptor for 17$\beta$-oestradiol from brain, pituitary and uterus of adult metoestrus female rats

| | | $K_{di}$ | | | | |
|---|---|---|---|---|---|---|
| *Unlabelled compound* | *Order of magnitude* | *Pituitary* | *Hypothalamus* | *Amygdaloid region* | *Cortex* | *Uterus* |
| MER-25 | $10^{-8}$ | $0.8 \pm 0.1$ | $1.0 \pm 0.2$ | $\sim 1$ | — | $\sim 1$ |
| 3$\alpha$ Diol | $10^{-7}$ | $4.1 \pm 0.8^*$ | $3.4 \pm 0.7^*$ | $9.8 \pm 1.9$ | — | $15.5 \pm 2.8$ |
| TClo | $10^{-8}$ | $4.7 \pm 0.7$ | $8.0 \pm 1.5$ | $7.2 \pm 2.1$ | — | $7.5 \pm 1.8$ |
| 3$\beta$ Diol | $10^{-8}$ | $4.0 \pm 0.2^*$ | $3.3 \pm 0.4^*$ | $5.2 \pm 0.7^*$ | — | $9.8 \pm 1.2$ |
| Cis Clo | $10^{-8}$ | $1.6 \pm 0.9$ | $1.3 \pm 0.5$ | $2.0 \pm 0.5$ | $1.6 \pm 0.3$ | $1.6 \pm 0.8$ |
| 17$\alpha$ $E_2$ | $10^{-8}$ | $2.2 \pm 0.2^*$ | $2.3 \pm 0.1^*$ | $5.0 \pm 0.8$ | $5.2 \pm 1.4$ | $4.1 \pm 0.5$ |
| $E_1$ | $10^{-8}$ | $1.9 \pm 0.1^*$ | $1.7 \pm 0.1$ | $4.3 \pm 0.5$ | $3.7 \pm 0.5$ | $3.3 \pm 0.4$ |
| $E_3$ | $10^{-9}$ | $1.6 \pm 0.1$ | $1.7 \pm 0.1$ | $2.4 \pm 0.3$ | $3.3 \pm 0.9$ | $2.1 \pm 0.3$ |
| Epi $E_3$ | $10^{-8}$ | $1.0 \pm 0.1$ | $1.3 \pm 0.1$ | $1.4 \pm 0.2$ | — | $1.9 \pm 0.4$ |
| Ru 2858 | $10^{-10}$ | $3.4 \pm 0.2$ | $7.1 \pm 0.7^*$ | $4.7 \pm 0.7$ | — | $4.6 \pm 0.3$ |
| 17$\beta$ $E_2$ | $10^{-10}$ | $1.0 \pm 0.1^*$ | $1.3 \pm 0.1$ | $1.3 \pm 0.1$ | $1.4 \pm 0.3$ | $1.5 \pm 0.2$ |
| DES | $10^{-11}$ | $2.8 \pm 0.4^*$ | $6.0 \pm 0.9$ | $4.9 \pm 0.8$ | $9.2 \pm 0.8$ | $8.9 \pm 2.2$ |

Each value is the mean (±s.e. mean) of 4 or 5 determinations.

* Significant difference from uterus $P < 0.01$.

of values contributing to the means. The results of these experiments with cytosols from metoestrus females are presented in Table 3. Values for affinity for each substance in uterine cytosol were compared with the values in cytosols from other tissues of female animals and, in paired *t*-tests $P < 0.01$ was taken as the criterion of significance. Significant differences from uterus were seen in 6 of the 12 compounds in pituitary and in 5 of the compounds in hypothalamus. Of these, 4 of the compounds are common to both hypothalamus and pituitary (3$\alpha$ androstan-diol, *trans*-clomiphene, 3$\beta$ androstan-diol and 17$\alpha$-oestradiol) and with a single exception (Ru 2858 in hypothalamus) the lower affinity was found in uterine cytosol. In contrast to pituitary and hypothalamus, no significant differences between the affinities in cortex and uterine cytosols were seen and there was only one compound with a different affinity in cytosol from amygdaloid region. Table 4 gives the values for the affinities estimated in cytosols from male animals. In only 3 of the 42 contrasts were the values significantly different ($P < 0.01$ in unpaired *t*-tests) from those in cytosols from the same tissues in females. Two of the contrasts concerned were in hypothalamus with compounds that in the female showed differential affinity between uterus and hypothalamus; the mean values for the affinities of the two compounds in male hypothalamic cytosol are remarkably close to the estimates using uterine cytosol.

## Discussion

The receptors in all the tissues studied are oestrogen selective and appear to be similar but are not necessarily identical. Similarities are found in the ranking orders of the affinities of the reaction of the compounds studied with the receptors which agree well with earlier reports of specificities of receptor binding in peripheral target tissues and with the known potencies (as oestrogens or anti-oestrogens) of the compounds used (Korenman, 1969; 1970; Raynaud, 1973). Dissociation constants of the reaction between [$^3$H]-17$\beta$-oestradiol and the high affinity sites are of the order $10^{-10}$ M which is appropriate to receptors mediating responses at physiological plasma concentrations of the steroid (Brown-Grant, Exley & Naftolin, 1973). Binding affinities for 17$\beta$-oestradiol measured by competition did not differ significantly from the direct estimates from the binding isotherm for [$^3$H]-oestradiol.

There is, nevertheless, some significant variation seen in the affinities of the reactions in different cytosols. Before concluding from the observed differences that there is evidence for heterogeneity of oestrogen receptors, possible experimental artefacts should be considered. The presence in cytosols of endogenous inhibitors would interfere with the binding reaction, and could account for differences between tissues but this seems an improbable source of artefact since if the receptors were homogeneous this should affect all estimates of $K_{di}$. Similarly, the influence of errors in the estimation of unbound oestradiol (*F*) can be discounted. It should be noted that the calculation of $K_{di}$ is based upon the assumption that receptor-inhibitor reaction had reached equilibrium within the incubation period, known to be adequate for the attainment of oestradiol-receptor equilibrium. The suggestion from these results of multiple forms of oestrogen receptors raises the possibility of the synthesis of tissue specific oestrogens or anti-oestrogens.

The results obtained with perphenazine and the two isomers of androstandiol are of particular interest in

**Table 4** Molar dissociation constants of oestrogen agonists and antagonists in reaction with cytosol high affinity receptor for 17$\beta$-oestradiol from brain and pituitary of adult male rats

| | | $K_{di}$ | | | |
|---|---|---|---|---|---|
| *Inhibitor* | *Order of magnitude* | *Pituitary* | *Hypothalamus* | *Amygdaloid region* | *Cortex* |
| MER-25 | $10^{-6}$ | ~1 | ~1 | ~1 | — |
| 3$\alpha$ diol | $10^{-7}$ | 2.6 ± 0.7 | 4.6 ± 1.4 | 4.4 ± 0.9 | — |
| TClo | $10^{-8}$ | 9.3 ± 2.1 | 10.2 ± 2.9 | 11.2 ± 4.5 | — |
| 3$\beta$ diol | $10^{-8}$ | 3.9 ± 0.3 | 3.5 ± 0.4 | 5.9 ± 0.9 | — |
| Cis Clo | $10^{-8}$ | 1.4 ± 0.4 | 2.0 ± 0.6 | 3.2 ± 1.5 | 2.6 ± 1.4 |
| 17$\alpha$ $E_2$ | $10^{-9}$ | 3.5 ± 0.7 | 4.0 ± 0.5* | 6.0 ± 0.7 | 3.9 ± 0.6 |
| $E_1$ | $10^{-8}$ | 3.0 ± 0.4 | 3.0 ± 0.4* | 6.0 ± 0.9 | 3.7 ± 0.7 |
| $E_3$ | $10^{-9}$ | 2.4 ± 0.5 | 1.9 ± 0.2 | 2.9 ± 1.2 | 2.6 ± 1.4 |
| Epi $E_3$ | $10^{-9}$ | 1.8 ± 0.5 | 0.9 ± 0.1 | 1.6 ± 0.5 | — |
| Ru 2858 | $10^{-10}$ | 2.6 ± 0.3 | 4.9 ± 0.4 | 2.3 ± 0.2* | — |
| 17$\beta$ $E_2$ | $10^{-10}$ | 1.2 ± 0.2 | 1.5 ± 0.2 | 2.0 ± 0.3 | 1.3 ± 0.2 |
| DES | $10^{-11}$ | 2.4 ± 0.5 | 5.9 ± 0.1 | 4.1 ± 0.1 | 8.1 ± 3.7 |

Each value is the mean (±s.e. mean) of 4–6 determinations.
* Significant difference from female $P < 0.01$.

view of the reported properties of these three compounds. Perphenazine, a lactogenic phenothiazine tranquillizer, has been reported to inhibit selectively the uptake of physiological doses of [$^3$H]-17$\beta$-oestradiol into both central and peripheral oestrogen target tissues, raising the possibility that the lactogenic effects of the drug might be mediated at least in part through binding to hypothalamic and pituitary oestrogen receptors (Shani *et al.*, 1971). In the present experiments no evidence was obtained for inhibition of [$^3$H]-17$\beta$-oestradiol binding to putative high affinity oestrogen receptor sites in any cytosol fraction, in the presence of a 5000-fold Molar excess of perphenazine. This suggests that the inhibition of [$^3$H]-17$\beta$-oestradiol uptake reported by Shani *et al.* (1971) does not represent direct interaction between perphenazine and available oestrogen receptor sites.

The ability of certain androgens, and in particular 5$\alpha$ androstan-3$\beta$,17$\beta$ diol, to induce oestrogen-like changes in the histology of the female reproductive tract has been known for some time (Williams-Ashman & Reddi, 1972). The present results provide evidence that 5$\alpha$ androstan-3$\beta$,17$\beta$ diol, and to a considerably lesser extent, its 3$\alpha$ isomer, may also interact directly with cytoplasmic oestrogen receptors, both in the uterus and in the central nervous system and the pituitary. In view of the fact that these two isomers of 5$\alpha$ androstandiol can be formed *in vivo* as metabolites of plasma androgen (the 3$\alpha$ epimer *in situ* in regions of the brain, and in the pituitary—Rommerts & van der Molen, 1971; Sholiton, Jones & Werk, 1972), these findings are of particular significance, since they suggest a mechanism, other than via aromatization to oestrogen, by which plasma androgen might impinge upon oestrogen receptor sites.

This work was carried out with support of grants from the Medical Research Council and the Wellcome Trust. N.J.M. was supported by a grant from Pfizers Limited.

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*(Received August 2, 1976.*
*Revised October 1, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 403–409

# EFFECTS OF ANTIDEPRESSANT DRUGS ON NORADRENALINE ACCUMULATION AND CONTRACTILE RESPONSES IN THE RAT ANOCOCCYGEUS MUSCLE

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1 The effect of a series of antidepressant drugs on noradrenaline accumulation was studied in the isolated anococcygeus muscle of the rat.

2 The most potent inhibitors of noradrenaline accumulation were nortriptyline, desipramine and protriptyline. Opipramol, trimipramine and iprindole were active only in high concentrations.

3 Contractions of the anococcygeus muscle produced by noradrenaline were strongly potentiated by nortriptyline, desipramine and protriptyline. Other uptake inhibitors were less active in potentiating the noradrenaline response.

4 Nortriptyline, in concentrations that potentiated the action of noradrenaline, reduced or abolished the response to tyramine.

## Introduction

The use of the isolated anococcygeus muscle of the rat in pharmacological research was first described by Gillespie (1972). This preparation appears to offer advantages over other sympathetically innervated isolated tissues and has been used successfully in studies of the actions of drugs acting on $\alpha$-adrenoceptors and on catecholamine release and uptake processes (Gillespie, 1972; Gibson & Gillespie, 1973; Gibson & Pollock, 1973; Nash, Gillespie & Robertson, 1974; Gillespie & McGrath, 1974; 1975).

In the present paper, we describe the effects of a series of antidepressant drugs on the accumulation of radioactive noradrenaline in the anococcygeus muscle. We have also studied the effects of the same drugs on the responses of the muscle to exogenously applied noradrenaline and to the indirectly-acting sympathomimetic, tyramine.

## Methods

Male rats (about 350 g) were used. The animals were killed and the anococcygeus muscles were dissected out as described by Gillespie (1972).

### *Effect of antidepressant drugs on noradrenaline accumulation*

The accumulation of radioactivity in the tissues was measured as follows. The anococcygeus muscles were removed from rats. The muscle was mounted on a wire frame under a constant tension of approximately 0.2 to 0.5 g and placed in a tube containing Krebs solution at 37°C. The solution was gassed with 95% $O_2$ and 5% $CO_2$. Tritiated noradrenaline, suitably diluted with unlabelled compound, was added to a final volume of 5 ml. The mixture was incubated for the appropriate length of time at 37°C, then blotted and washed for 10 min in 5 ml of Krebs solution. The muscle was blotted dry, cut into 4 approximately equal pieces which were weighed. Each portion of muscle was placed in a test tube with 0.4 ml of 'protosolve' (NaOH 120 g in one litre methanol). When the tissue had dissolved, 10 ml of scintillation fluid and 0.25 ml of glacial acetic acid were added. The radioactivity was counted in a Phillips liquid scintillation counter. The radioactivity remaining in the medium after the incubation was determined by taking a 0.1 ml sample of medium and adding 0.9 ml of distilled water together with 10 ml of scintillation fluid and counting the radioactivity. The accumulation of radioactivity, expressed as nmol noradrenaline/ g tissue, and the tissue:medium ratios were then calculated.

Parallel experiments were performed in which the effect of various antidepressant drugs on the noradrenaline accumulation was studied. In these experiments the incubations were carried out in Krebs solution containing different concentrations of drugs. Inhibition of noradrenaline accumulation was expressed as a percentage of control values. Dose-response curves were plotted of % inhibition of noradrenaline accumulation against log M concentration of uptake inhibitor. The $IC_{50}$ values (concentration causing 50% inhibition of noradrenaline accumulation) for each compound were calculated from regression lines.

### *Chromatography*

Anococcygeus muscles were incubated for 20 min in 5 ml of Krebs solution containing 0.6 nmol of tritiated noradrenaline, then blotted and washed as previously described. After weighing, 4 muscles were pooled, cut into small pieces and ground in 0.35 ml of ice cold acetic acid (0.1 mol/l) with a glass grinder. The mixture was centrifuged and 30 µl of the supernatant was run on a thin layer chromatography plate using either *n*-butanol:water:acetic acid (12:5:3: by vol) or isopropanol:2M HCl (65:35 v/v) as the solvent system. The radioactivity of 1 cm strips of the chromatography plate was then determined. Unlabelled noradrenaline together with the major noradrenaline metabolites, was run concurrently in the same solvent systems. The position of the unlabelled material was located by the use of diazotised *p*-nitroaniline as described by Fleming & Clark (1970).

### *Drugs*

(—)-[7-$^3$H]-noradrenaline hydrochloride (specific activity 10.3 Ci/mmol) was obtained from Amersham. Unlabelled (—)-noradrenaline bitartrate (Koch-Light), together with 200 ng (in 10 µl) of [$^3$H]-noradrenaline was made up to 1 ml in tartaric acid solution (1.5 mmol/l): 0.1 ml of this solution was made up to 5 ml with Krebs solution to give a final noradrenaline concentration of 61 pmol/ml. This final solution was kept under liquid nitrogen until required for use. Other concentrations were prepared accordingly by varying the amount of unlabelled noradrenaline added.

The other drugs used were tyramine hydrochloride, acetylcholine chloride (Koch-Light); carbachol (BDH); DL-3,4-dihydroxymandelic acid, DL-methoxy-4-hydroxymandelic acid DL-normetanephrine (Aldrich); amitriptyline hydrochloride, maprotiline hydrochloride, protriptyline hydrochloride, imipramine hydrochloride, desipramine hydrochloride, clomipramine hydrochloride, opipramol dihydrochloride (Ciba-Geigy); nortriptyline hydrochloride (Eli Lilly); doxepin hydrochloride (Pfizer) iprindole (John Wyeth).

The Krebs solution used had the following composition (g/l): $CaCl_2.6H_2O$ 0.55, KCl 0.35, $KH_2PO_4$ 0.16, $MgSO_4.7H_2O$ 0.29, $NaHCO_3$ 2.1, NaCl 7.1 and glucose 1.0.

### *Dose-response curves on the anococcygeus muscle*

Individual anococcygeus muscles were set up, with a resting tension of 0.2 to 0.5 g, in a 10 ml bath containing Krebs solution at 37°C. The preparation was gassed with 95% $O_2$ and 5% $CO_2$. Contractions of the muscle were recorded with an isometric transducer and a Servoscribe pen recorder.

Drugs were added to the preparation in a small volume (less than 0.3 ml) to give the final bath concentrations indicated. Dose-response curves were obtained as described by Gibson & Pollock (1973). Responses were expressed as a percentage of the maximum noradrenaline response which was between 3 and 7 g tension. A regression line was calculated for each dose-response curve over the range 20 to 80% of the maximum response. The $pD_2$ values (negative logarithm of M concentration of agonist producing 50% of maximum response) were obtained as described by Gibson & Pollock (1973). Additional dose-response curves were obtained to acetylcholine or carbachol.

When studying the effects of antidepressant drugs on the response, these drugs were present in the Krebs solution throughout the experiment; the tissue was allowed to equilibrate with the drug for 30 min before the experiment was started. $pD_2$ values for noradrenaline and tyramine in the presence of various antidepressant drugs were obtained in the usual way. Statistical evaluation was carried out using an unpaired *t* test.

The ability of drugs to potentiate the actions of noradrenaline are expressed as the dose-ratios (potentiation), which were obtained by taking the antilogarithm of the difference between the $pD_2$ values for noradrenaline obtained in the presence and absence of antidepressant drug. The ability of the same drugs to inhibit the response to tyramine is expressed, where possible, as the dose-ratio (inhibition), which was calculated in an analogous way.

## Results

### *Time course of noradrenaline accumulation*

The accumulation of radioactivity in the anococcygeus muscle following different times of incubation was measured at 3 different concentrations of noradrenaline (31, 61 and 122 pmol/ml). With these low concentrations uptake is likely to be mainly into adrenergic nerves, with no accumulation within smooth muscle (Nash *et al.*, 1974). The results obtained in these experiments are illustrated in Figure 1 where maximum tissue:medium ratios of $5.4 \pm 0.8$ (s.e. mean : $n=4$) were obtained with 61 pmol/ml. It can be seen that accumulation of radioactivity in the preparation increased with time and with increasing noradrenaline concentrations in the incubation fluid.

### *Identification of noradrenaline*

In 4 separate experiments muscles that had been incubated with tritiated noradrenaline for 20 min were

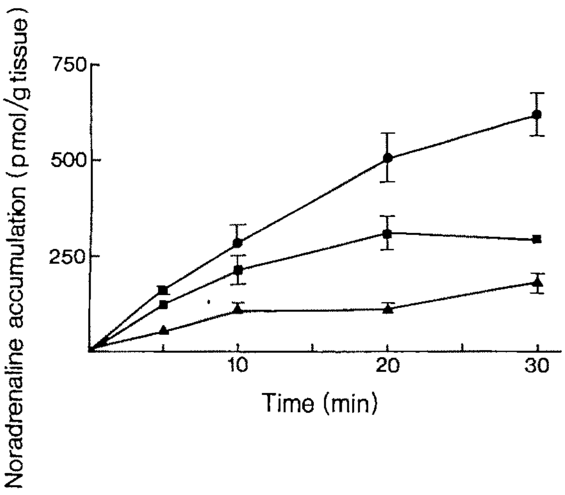

**Figure 1** Time course of accumulation of radioactivity in rat anococcygeus muscle following incubation in tritiated noradrenaline at the following concentrations: (▲) 31 pmol/ml; (■) 61 pmol/ml; (●) 122 pmol/ml. Each point is the mean of 4 observations. Vertical lines show s.e. means.

homogenized and an aliquot of the muscle homogenate was run on thin layer chromatograms in two separate solvent systems. In all cases radioactivity eluted from the plate corresponded to authentic noradrenaline, there being no evidence for the presence of any metabolites of noradrenaline (Figure 2).

**Table 1** Inhibition of noradrenaline uptake by drugs. Potency is expressed as $IC_{50}$ values and as the equipotent molar ratio (epmr) which is $IC_{50}$ for drug/$IC_{50}$ nortriptyline

| *Drug* | *$IC_{50}$ (M)* | *epmr* |
|---|---|---|
| Nortriptyline | $1.0 \times 10^{-8}$ | 1 |
| Desipramine | $4.0 \times 10^{-8}$ | 4 |
| Protriptyline | $5.9 \times 10^{-8}$ | 6 |
| Imipramine | $2.9 \times 10^{-7}$ | 29 |
| Doxepin | $8.9 \times 10^{-7}$ | 89 |
| Cocaine | $9.2 \times 10^{-7}$ | 92 |
| Clomipramine | $1.2 \times 10^{-6}$ | 120 |
| Maprotiline | $1.3 \times 10^{-6}$ | 130 |
| Amitriptyline | $2.3 \times 10^{-6}$ | 230 |
| Opipramol | $1.9 \times 10^{-5}$ | 1900 |
| Trimipramine | $2.9 \times 10^{-5}$ | 2900 |
| Iprindole | $5.6 \times 10^{-5}$ | 5600 |

Values were calculated from regression lines and involved at least four separate determinations for each compound.

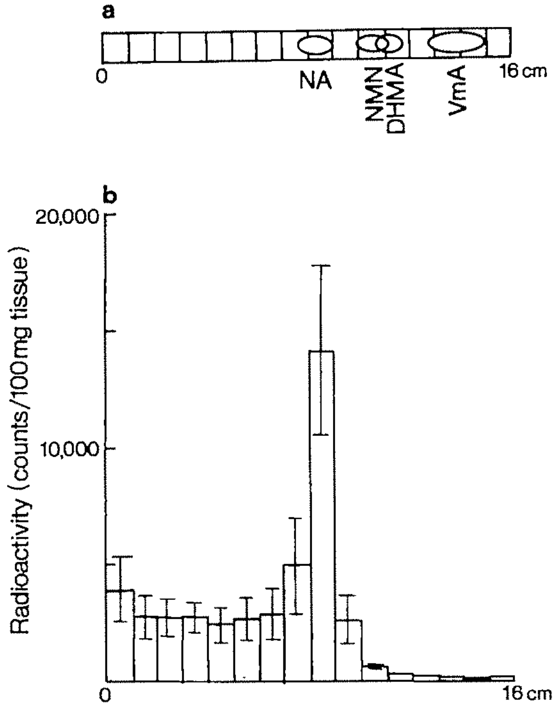

**Figure 2** (a) Thin layer chromatogram of noradrenaline (NA) and some NA metabolites. Solvent system *n*-butanol:$H_2O$:acetic acid. NMN=normetanephrine; DHMA=3,4-dihydroxymandelic acid; VMA=3methoxy-4-hydroxymandelic acid. (b) Thin layer chromatogram of radioactivity extracted from anococcygeus muscle following incubation with tritiated noradrenaline. Solvent system as in (a). Note that the major peak of radioactivity corresponds to authentic noradrenaline. Values are the mean of 4 observations. Vertical lines show s.e. means. In (a) and (b) each plate was allowed to run 16 cm.

### *Inhibition of noradrenaline accumulation by drugs*

The most potent inhibitors of noradrenaline accumulation were nortriptyline, desipramine and protriptyline. Each of these compounds caused 50% inhibition of uptake at concentrations of less than $10^{-7}$M. The dose-response curves for inhibition of accumulation by nortriptyline, amitriptyline and iprindole are shown in Figure 3. The $IC_{50}$ values, obtained from regression lines, for all compounds tested are shown in Table 1.

### *Effects of drugs on the response to noradrenaline*

Noradrenaline caused powerful contractions of the anococcygeus muscle, as previously described

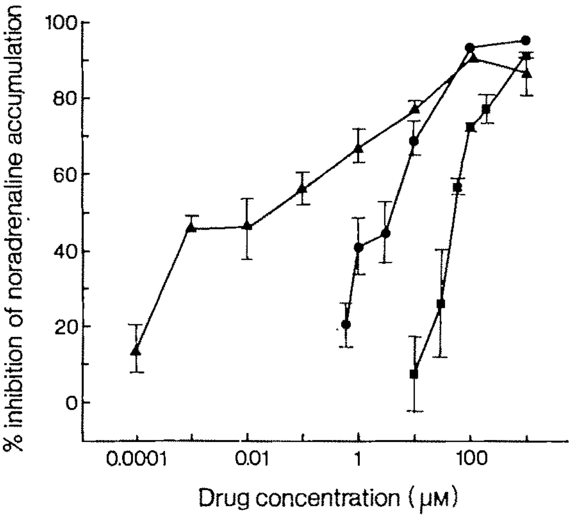

**Figure 3** Dose-response curves for the inhibition by drugs of noradrenaline accumulation in rat anococcygeus muscle: (▲) inhibition by nortriptyline; (●) inhibition by amitriptyline; (■) inhibition by iprindole. Each point is the mean of 4 observations. Vertical lines show s.e. means. Noradrenaline accumulation was calculated as % of control accumulation occurring in the absence of drugs after incubation for 20 min in [$^3$H]-noradrenaline (61 pmol/ml).

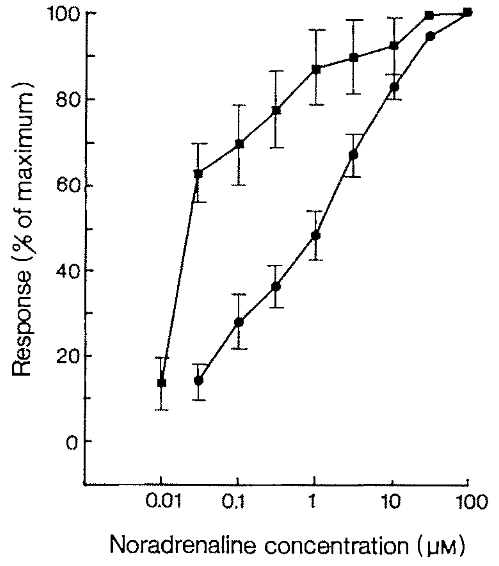

**Figure 4** Effect of nortriptyline on the response to noradrenaline of the rat anococcygeus muscle. Responses are expressed as a percentage of maximum response: (●) contractions produced by noradrenaline alone; (■) effect of noradrenaline in the presence of $10^{-5}$ M nortriptyline. Each point is the mean of 4–6 observations. Vertical lines show s.e. means.

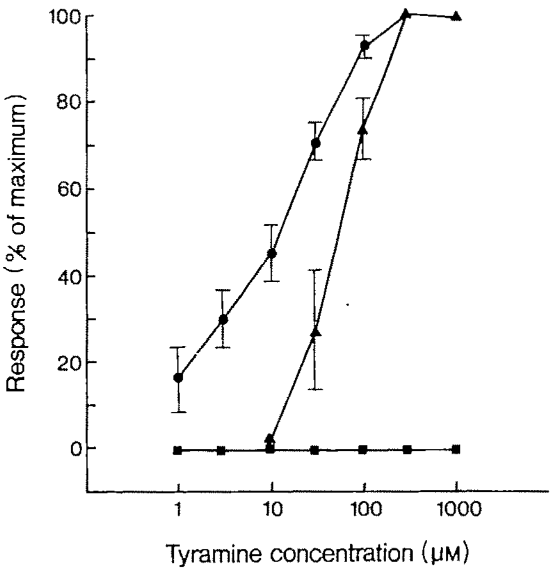

**Figure 5** Effect of nortriptyline on the response to tyramine of the rat anococcygeus muscle. Responses are expressed as a percentage of the maximum response which was the same as that produced by $10^{-4}$ M noradrenaline. (●) Contractions produced by tyramine alone; (▲) effect of tyramine in the presence of $10^{-7}$ M nortriptyline. In the presence of $10^{-5}$ M nortriptyline (■) the tyramine response was completely abolished. Each point is the mean of 4–6 observations. Vertical lines show s.e. means.

(Gillespie, 1972). The $pD_2$ value for noradrenaline was $6.2 \pm 0.2$ (s.e. mean, $n = 10$), and maximum contractions were produced at $10^{-4}$ M. None of the antidepressant drugs caused contraction of the muscle in the concentrations tested.

The most active inhibitors of noradrenaline accumulation were also the most potent in terms of their ability to potentiate the actions of exogenously applied noradrenaline (Table 2). The degree of potentiation produced was usually greatest at a tricyclic antidepressant concentration of $10^{-6}$ or $10^{-7}$M. The effect of nortriptyline on the noradrenaline response is illustrated in Figure 4. Maximum potentiation of the noradrenaline response was produced by $10^{-7}$ M nortriptyline.

Nortriptyline ($10^{-7}$ M) reduced the response of the muscle to tyramine (Figure 5). At $10^{-5}$ M nortriptyline the response to tyramine (up to $10^{-3}$ M) was completely abolished, whereas the response to noradrenaline was still potentiated, although to a lesser extent than at $10^{-7}$ M. None of the drugs tested caused potentiation of the tyramine response. Clomipramine and opipramol had no potentiating actions but at a concentration of $10^{-5}$ M each of these compounds reduced the response produced by noradrenaline.

The effects of the other compounds tested on the responses of the muscle to noradrenaline and tyramine are summarized in Table 2.

### *Effects of drugs on the response to acetylcholine and carbachol*

Acetylcholine causes contraction of the anococcygeus muscle (Gillespie, 1972). In the present study the acetylcholine dose-response curves were not significantly shifted by any of the compounds listed in Table 2 (each at $10^{-6}$ M).

At a higher concentration ($10^{-5}$ M) nortriptyline, protriptyline and doxepin antagonized the action of acetylcholine. Iprindole ($10^{-5}$ M) potentiated responses to acetylcholine or carbachol.

## Discussion

There are two main processes by which noradrenaline can be taken up by tissues. The neuronal system (Uptake$_1$) has a high affinity for noradrenaline. In addition, there is the lower affinity extraneuronal system, designated Uptake$_2$. (See reviews by Iversen, 1967; 1971a & b.)

In a recent study Nash *et al.* (1974) provided strong evidence that at noradrenaline concentrations below 2 μg/ml the rat anococcygeus muscle accumulates noradrenaline mainly into adrenergic nerves, with little contribution by Uptake$_2$. In the present study we have used low concentrations of noradrenaline in order to study mainly the neuronal uptake system. In our chromatographic experiments we have shown that at these low noradrenaline concentrations, all of the radioactivity retained in the muscle behaves as

**Table 2** Potency of drugs in terms of their ability to potentiate the response to noradrenaline and inhibit the response to tyramine on the rat anococcygeus muscle.

| *Drug* | *Concn (M)* | *Noradrenaline response Dose-ratio (potentiation)* | *Tyramine response Dose-ratio (inhibition)* |
|---|---|---|---|
| Nortriptyline | $10^{-5}$ | 25*** | CI |
| | $10^{-6}$ | 26** | 5* |
| | $10^{-7}$ | 69** | 7** |
| Protriptyline | $10^{-5}$ | 0 | CI |
| | $10^{-6}$ | 23** | 12** |
| | $10^{-7}$ | 13** | 0 |
| Desipramine | $10^{-5}$ | 0 | CI |
| | $10^{-6}$ | 9** | 6* |
| | $10^{-7}$ | 20*** | 0 |
| Imipramine | $10^{-5}$ | Redn | 29*** |
| | $10^{-6}$ | 14*** | 0 |
| | $10^{-7}$ | 0 | 0 |
| Doxepin | $10^{-5}$ | 0 | CI |
| | $10^{-6}$ | 13** | 3* |
| | $10^{-7}$ | 0 | 0 |
| Maprotiline | $10^{-5}$ | 5** | CI |
| | $10^{-6}$ | 0 | 0 |
| Amitriptyline | $10^{-5}$ | 0 | CI |
| | $10^{-6}$ | 7** | 0 |
| Trimipramine | $10^{-5}$ | 0 | CI |
| | $10^{-6}$ | 0 | 6** |
| Iprindole | $10^{-5}$ | 0 | 5 |
| | $10^{-6}$ | 5** | 0 |

Noradrenaline potentiation is expressed as dose-ratio (potentiation) and tyramine inhibition as dose-ratio (inhibition) (seeMethods section). Values quoted are the mean of at least 4 determinations.
Levels of significance (unpaired *t* test) when compared with control responses for noradrenaline or tyramine: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.
0=no significant difference from controls; Redn=noradrenaline response was reduced; CI=complete or almost complete inhibition of tyramine response (thus not possible to calculate dose-ratios).

authentic noradrenaline, with no detectable presence of noradrenaline metabolites.

Many of the tricyclic antidepressant drugs are potent inhibitors of the neuronal uptake of noradrenaline (reviews by Biel & Bopp, 1974; Iversen, 1971a). Desipramine has been shown to be potent as a noradrenaline uptake inhibitor in rabbit aortic strips (Maxwell, Chaplin, Eckhardt, Soares, & Hite, 1970), rat brain slices (Salama, Insalaco & Maxwell, 1971), rat heart (Iversen, 1967) and in synaptosomes from rat brain (Horn, Coyle & Snyder, 1971; Ross & Renyi, 1975). Nortriptyline is similarly a potent noradrenaline uptake inhibitor, being only about 3 or 4 times less active than desipramine on rat brain synaptosomes and on rat heart (Iversen, 1967; Ross & Renyi, 1975), although slightly lower values for the potency of this compound have been reported by Horn *et al.* (1971) in their experiments on rat brain synaptosomes and by Maxwell *et al.* (1970).

In the results reported in the present paper nortriptyline and desipramine were powerful inhibitors of noradrenaline accumulation, nortriptyline being slightly more active than desipramine. The least active compound in our studies was iprindole, which was more than 5000 times less active than nortriptyline. This is in agreement with the work of others, who have shown that iprindole is weakly active as a noradrenaline uptake inhibitor (Gluckman & Baum, 1969; Lemberger, Sernatinger & Kuntzman, 1970; Lahti & Maickel, 1971; Ross, Renyi & Ogren, 1971). In spite of its low potency as an inhibitor of biogenic amine uptake, iprindole is a clinically effective antidepressant agent (for references see Biel & Bopp, 1974).

The most potent inhibitors of noradrenaline accumulation caused a large potentiation of the noradrenaline response in this tissue. That this potentiation is specific for the noradrenaline system is indicated by the fact that, with the exception of iprindole, none of the compounds tested caused potentiation of the response to acetylcholine or carbachol. Thus it is likely that, whereas iprindole may have a different mechanism of action, the other drugs potentiate the noradrenaline response by blocking noradrenaline uptake. A more direct way to study this would be to investigate the effects of the antidepressant drugs on an $\alpha$-agonist which is not a substrate for uptake (Gibson & Pollock, 1973). However, this approach presents difficulties since drugs like oxymetazoline and methoxamine, which are not taken up presynaptically in this tissue, are nevertheless much more susceptible to $\alpha$-blocking activity than is noradrenaline (Doggrell & Woodruff, unpublished observations).

The use of noradrenaline uptake inhibitors provides a means of distinguishing between directly-acting and indirectly-acting sympathomimetic amines. Thus in many tissues cocaine potentiates the actions of noradrenaline and some other directly-acting agonists but antagonizes the action of the indirectly-acting tyramine (see reviews by Iversen, 1971a; Trendelenburg, 1972).

In the anococcygeus muscle preparation cocaine potentiates the response to noradrenaline but the usefulness of this compound is limited by the fact that it releases noradrenaline and itself causes the muscle to contract (Gibson & Pollock, 1973; Gillespie & McGrath, 1974).

The use of tricyclic antidepressant drugs as uptake inhibitors is hampered in tissues containing $\alpha$-adrenoceptors (Iversen, 1971a). Barnett, Symchowicz & Taber (1968) and Barnett, Staub & Symchowicz (1969), using the rat isolated vas deferens, have suggested that the antagonism of the tyramine response produced by imipramine and desipramine is due to the $\alpha$-blocking activity of the drugs rather than to their ability to inhibit uptake. Hughes, Kneen & Main (1974), using the vas deferens of mouse and rat, have shown the effect of desipramine on the response of the tissues to noradrenaline is a balance between potentiation due to uptake block and antagonism due to receptor block. Such a balance is similarly indicated in our experiments. For example, desipramine, imipramine, doxepin and amitriptyline each caused potentiation of the noradrenaline response at $10^{-6}$ M but no potentiation at $10^{-5}$ M, presumably because at the high concentration the $\alpha$-blocking activity of the compounds becomes significant. Nortriptyline has less $\alpha$-blocking activity than desipramine and many other tricyclic antidepressants. This is shown in the experiments of Sturman (1971) who measured the potentiation produced by a series of tricyclic compounds on the response of the cat nictitating membrane to noradrenaline; nortriptyline caused a greater degree of potentiation than desipramine, imipramine or amitriptyline. The relatively weak $\alpha$-blocking activity of nortriptyline is apparent in our experiments where in the presence of $10^{-5}$ M nortriptyline the noradrenaline response is still greatly potentiated.

There is evidence that the actions of tyramine on the anococcygeus muscle are mediated indirectly by the release of noradrenaline, since the responses are abolished by pretreatment with 6-hydroxydopamine (Gillespie & McGrath, 1975). With many of the uptake inhibitors used in the present study, the inhibition of the tyramine response is probably due to a combination of uptake inhibition and $\alpha$-blocking activity. However, it is clear that using nortriptyline it is possible to distinguish between the action of noradrenaline and that of the indirectly-acting tyramine on this preparation.

We are grateful to the following for gifts of drugs: May & Baker Ltd., Ciba-Geigy Ltd., Eli Lilly Ltd., Pfizer Ltd. and John Wyeth & Brother Ltd. One of us (S.A.D.) was supported by a M.R.C. research award.

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*(Received August 2, 1976.*
*Revised October 26, 1976.)*

*Br. J. Pharmac.* (1977), 59, 411–417

# EFFECTS OF D-600 AND ITS OPTICAL ISOMERS ON FORCE OF CONTRACTION IN CAT PAPILLARY MUSCLES AND GUINEA-PIG AURICLES

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1 (–)-D-600 and (+)-D-600 exerted concentration-dependent negative inotropic effects in papillary muscles from cats, the potency of (–)-D-600 being about 100 times greater than that of (+)-D-600. The action was more pronounced at high than at low frequencies of stimulation. Time to peak force, relaxation time and therefore also total duration of contraction were not significantly affected by the drugs.

2 The negative inotropic effects of both drugs were not reversible after washing in drug-free solution for 60 min at all concentrations tested.

3 The same negative inotropic effects were seen in guinea-pig left auricles with the racemic mixture of both isomers.

4 Uptake measurements of D-600-[nitrile-$^{14}$C] in isolated left auricles of guinea-pigs showed the drug to be accumulated in the myocardial tissue; tissue-medium ratios from 1.25 to 6.0 were found at exposure times from 1 to 100 minutes. Preparations which were loaded first with D-600-[nitrile-$^{14}$C] for 100 min and then washed in drug-free solution for different times lost up to about 80% of the initial radioactivity, whereas the depression of force of contraction was not reversible.

5 The results show that there are marked quantitative differences between the actions of (–)-D-600 and (+)-D-600 on isometric force of contraction in cat papillary muscles. Although the time course of the uptake of D-600-[nitrile-$^{14}$C] and the development of the negative inotropy in guinea-pig left auricles were reasonably well related, the magnitude of the negative inotropy does not seem to be dependent on the total tissue concentration once the negative inotropic effect has been fully developed.

## Introduction

A growing body of evidence suggests that there is a close relationship between the slow inward calcium current ($I_{Ca}$) during the action potential and twitch tension in mammalian ventricular muscle (Beeler & Reuter, 1970; Bassingthwaighte & Reuter, 1972; Trautwein, McDonald & Tripathi, 1975). The organic compounds verapamil and its methoxyderivative D-600 were said to diminish force of contraction in guinea-pig papillary muscles (Fleckenstein, Tritthart, Fleckenstein, Herbst & Grün, 1969) and to reduce specifically $I_{Ca}$ in cat papillary muscles (Kohlhardt, Bauer, Krause & Fleckenstein, 1972). These organic compounds are often used to identify transmembrane calcium movements; verapamil has been used to obtain further information about the mechanism of physiological and pharmacological effects on myocardial contractility (McCans, Lindenmayer, Munson, Evans & Schwartz, 1974).

Apart from the fact that the statement of a specific inhibition of $I_{Ca}$ by verapamil or D-600 seems to require modification (Kass & Tsien, 1975), before these substances are used as tools for studying myocardial contractility their actions on force of contraction in the heart should be characterized more precisely to include concentration-response relationships, the time course of the negative inotropy, and a quantification of the reversibility or irreversibility of the effects.

In the present paper, the action of D-600 and its optical isomers on force of contraction either in cat papillary muscles or in guinea-pig auricles is described, including the effects on isometric twitch tension, time to peak force and relaxation time. There seems to be a correlation between the time course of the uptake of D-600-[nitrile-$^{14}$C] and the development of the negative inotropy, whereas no such correlation could be found between the recovery of force of contraction and release of D-600-[nitrile-$^{14}$C] when the preparations were first exposed to the drug and then washed in drug-free solution.

A preliminary account of this work has been published (Ludwig & Nawrath, 1976).

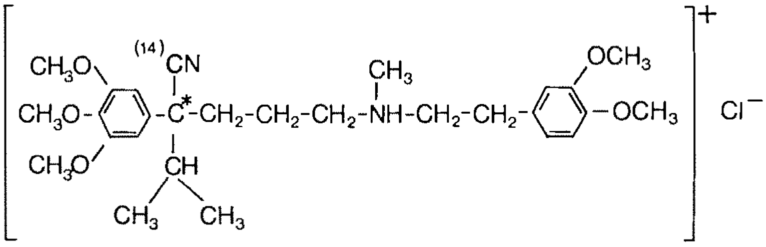

Figure 1 Structural formula of D-600-hydrochloride (α-isopropyl-α-[(*N*-methyl-*N*-homoveratryl)-γ-aminopropyl]-3,4,5-trimethoxyphenylacetonitrile-hydrochloride). The optically active C-atom is marked by an asterisk. The radioactive molecule is $^{14}$C-labelled at the nitrile group.

## Methods

The hearts of young cats of either sex (body weight: 1–2 kg) were removed following sodium pentobarbitone (35 mg/kg) anaesthesia. The right ventricles were opened in a dissection chamber containing warm, oxygenated Tyrode solution. Papillary muscles were isolated by tying both ends with fine silk sutures and then dissecting them from the ventricular wall. The preparations were attached to a muscle holder assembled with a bipolar platinum stimulating electrode and mounted vertically in a muscle chamber containing 5 ml Tyrode solution. The upper, tendinous end of the preparations was connected to an inductive force displacement transducer (built in our workshop by H. Fleck) by means of a stainless steel wire. The muscles were driven electrically by rectangular pulses (Grass stimulator S 4; intensity 10–20% above threshold) of 2 ms duration at 0.1 to 1.0 Hz. Tension was recorded under isometric conditions at the apex of the preload active tension curve and monitored on a Hellige paper recorder.

The hearts of guinea-pigs (body weight: 200–300 g) were obtained from animals killed by a blow on the head. The left auricles were removed from the rest of the heart, mounted as described for the papillary muscles from cats, and driven at 2 Hz.

The Tyrode solution used was prepared with distilled deionized water and had the following composition (mM): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, $NaH_2PO_4$ 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 1.8 and glucose 5.5. The Tyrode solution in the muscle chambers was continuously gassed with 95% $O_2$ and 5% $CO_2$; the temperature was maintained constant at 35°C. The experimental set up permitted a rapid exchange (1–2 s) of one solution for another.

### *Drugs*

(−)-D-600, (+)-D-600, (±)-D-600, (−)-verapamil and (+)-verapamil were supplied by Knoll AG, Ludwigshafen. The abbreviated form D-600 always refers to the racemic mixture of the drug: (±)-D-600. In a series of experiments, D-600-[nitrile-$^{14}$C] (purity 99·7%; Knoll AG, Ludwigshafen) was used. The structural formula of D-600 is given in Figure 1. The asymmetric C-atom is indicated by an asterisk; verapamil is the corresponding compound but with only two methoxy groups on the left hand benzene ring. The absolute configuration of (−)-verapamil has been described as L-, and of (+)-verapamil as D-form of the optically active isomers (Ramuz, 1975). The purity of the optically active isomers is 100% (Dengel & Oberdorf, Knoll AG, personal communication). The pH of the test solutions was not changed in the presence of these drugs and ranged from 7.3 to 7.4. All drugs were freshly prepared in distilled deionized water and 0.1 ml of the corresponding stock solutions were injected into the muscle chamber containing 5 ml Tyrode solution.

### *Experimental procedure*

Papillary muscles were first allowed to stabilize for 60 min at 0.5 Hz. Then the preparations were driven for 5 min at each of a number of different frequencies (0.1, 0.25, 0.5, 0.75, 1.0 Hz). After this period, the driving frequency was set at 0.5 Hz and the muscles were exposed to drugs when the twitch height was steady (within 5 to 10 minutes). The drug effect was monitored for 30 min and force-frequency relationships were obtained as described for the control period. Finally, the muscles were washed 3 times and tension was monitored for a further 60 minutes. At the end of each experiment, the preparations were measured in length with a microscope, then blotted with filter paper for 90 s under constant pressure (280 g) and weighed. The cross sectional area of the papillary muscles was determined according to Koch-Weser (1963). The mean cross sectional area ± s.d. of 107 preparations was 0.55 ± 0.32 mm$^2$. Individual isometric contraction curves were displayed at higher paper speed

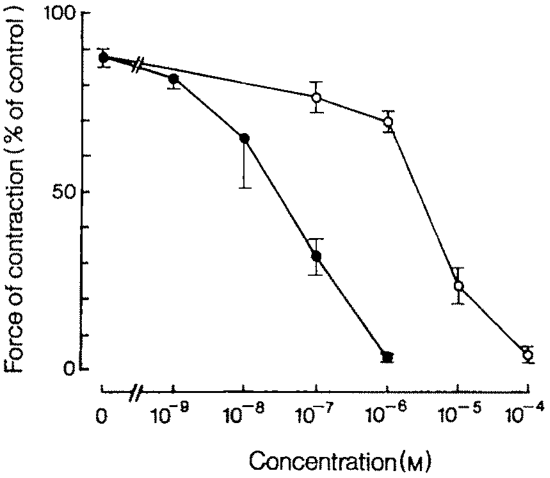

**Figure 2** Concentration-response relationships of (−)-D-600 (●) and (+)-D-600 (○) in cat papillary muscles. Values are means of 6 preparations. Vertical bars show s.e. means. Both isomers exerted a concentration-dependent negative inotropic effect: $ED_{50}$ of (−)-D-600 was approx. $3 \times 10^{-8}$ M, of (+)-D-600 approx. $3 \times 10^{-6}$ M.

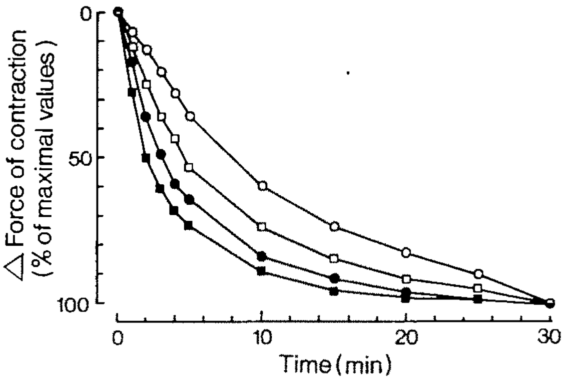

**Figure 3** Time course of the negative inotropic effects of (−)-D-600 and (+)-D-600 (same preparations as in Figure 2). The drug effects were evaluated as differences (△) in force of contraction between control and test values and expressed as percentages of the maximal values reached after 30 minutes. With both isomers the effects developed faster at high than at low concentrations. Standard errors are omitted for clarity. Concentrations: (○) $10^{-8}$ M, (●) $10^{-6}$ M of (−)-D-600; (□) $10^{-6}$ M, (■) $10^{-4}$ M of (+)-D-600.

(100 mm/s) or photographed from a dual beam oscilloscope (Tektronix 502 A). These records were analyzed with regard to their height, time to peak force and relaxation time according to Reiter & Stickel (1968). Results are expressed as means ± s.e.

### *Tracer experiments*

Experiments with D-600 or D-600-[nitrile-$^{14}$C] were done in guinea-pig left auricles. The muscles were first allowed to stabilize for 60 min and then exposed for different times (1–100 min) to the labelled racemic mixture of D-600. In another series of experiments, the auricles were first loaded with D-600-[nitrile-$^{14}$C] for 100 min and then washed in drug-free solution for different times (1–300 minutes). At the end of each experiment, the auricles were removed and after discarding the cut edges and sites of attachment they were blotted on filter paper for 90 s under constant pressure (280 g) and weighed. The preparations were then dissolved in scintillation vials by the addition of tissue solubilizer (Soluene, Packard), 0.5 ml and heating to 65°C for 3 hours. To the resulting solution 1N HCl, 1 ml and liquid scintillator (Unisolve, Zinsser), 10 ml were added. The samples were placed in a Tri-Carb liquid scintillation spectrometer (Packard, model 3380) and radioactivity was counted 3 h later.

## Results

### *Action of the optical isomers of D-600 on force of contraction in cat papillary muscles*

The inotropic effects of (−)-D-600 and (+)-D-600 in cat papillary muscles (at 0.5 Hz, 30 min after addition of the drug) are shown in Figure 2. Each concentration was tested in individual preparations. With both drugs, a concentration-dependent negative inotropic effect was observed. The concentration-response relationships suggest that there are marked quantitative differences in the action of these drugs, the potency of (−)-D-600 being about 100 times greater than that of (+)-D-600. Time to peak tension, relaxation time and therefore also total duration of contraction remained virtually unchanged with both drugs at all concentrations tested. After a 60 min washing period in drug-free solution force of contraction ($F_c$) remained unchanged or was even slightly smaller than 30 min after drug addition. Therefore, the negative inotropic effects of both (−)-D-600 and (+)-D-600 are thought to be completely irreversible. Figure 3 shows the time course of the action of the optical isomers of D-600, each at two concentrations. The time-dependent differences (Δ) between control $F_c$ and $F_c$ after drug addition are expressed as percentage values of maximal values measured 30 min after drug exposure. This type of

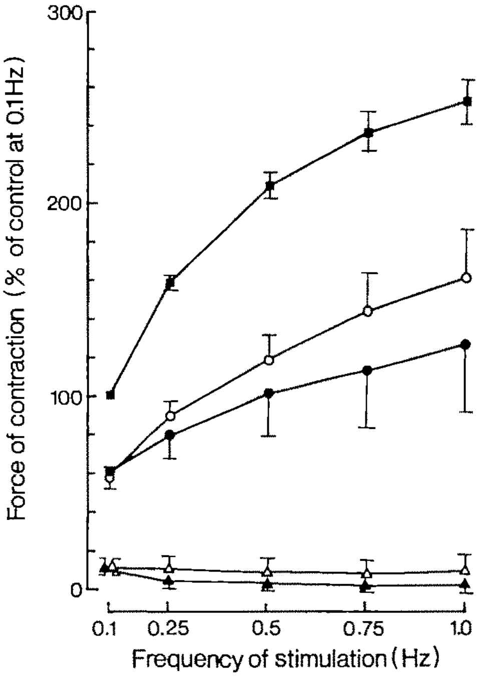

**Figure 4** Force of contraction ($F_c$) of controls and preparations either treated with (−)-D-600 or (+)-D-600 at various frequencies of stimulation (same preparations as in Figure 2). $F_c$ of untreated controls at 0.1 Hz is taken as 100% and all other values are expressed as percentages. $F_c$ of untreated controls increased to about 250% at 1.0 Hz. This 'positive staircase phenomenon was diminished or abolished in a concentration-dependent way by (−)-D-600 and (+)-D-600: (■) control ($n$=107); (●) $10^{-8}$ M, (▲) $10^{-6}$ M of (−)-D-600; (○) $10^{-6}$ M, (△) $10^{-4}$ M of (+)-D-600.

evaluation demonstrates that the development of the negative inotropy becomes faster and the steady state drug effect is reached more rapidly with high concentrations than with low ones. This holds true for both (—)-D-600 and (+)-D-600 and, again, it is evident that both drugs show quantitative rather than qualitative differences in action.

Figure 4 shows force-frequency relationships of controls and preparations treated either with (—)-D-600 or (+)-D-600. Control values at 0.1 Hz were taken as 100% and all other values are given as percentages. The upper curve of untreated control muscles demonstrates the well known staircase phenomenon of increasing $F_c$ when the driving rate is increased (Bowditch, 1871). This phenomenon was diminished in a concentration-dependent way and finally abolished with both (—)-D-600 and (+)-D-600. In other words, at low concentrations, the negative inotropic effect was dependent on the driving rate; at higher concentrations, when the staircase phenomenon was abolished, $F_c$ was diminished to the same final value at all frequencies of stimulation tested. Figure 4 also demonstrates only quantitative differences between the action of (—)-D-600 and (+)-D-600, the factor of potency again being about 100.

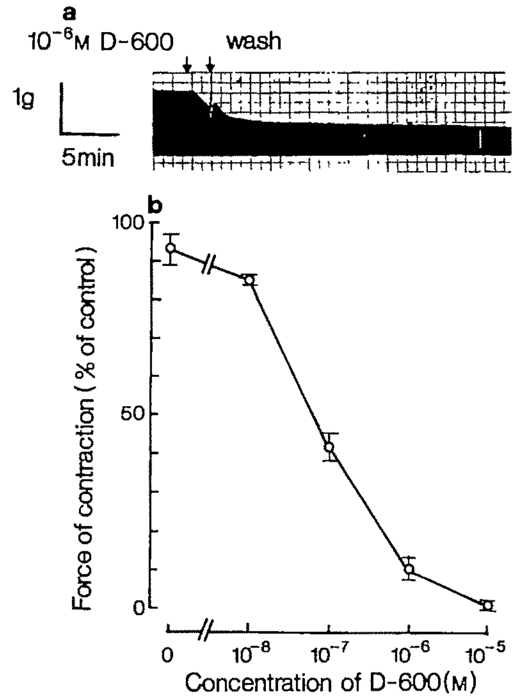

**Figure 5** (a) Original tracing of a guinea-pig left auricle exposed to $10^{-6}$ M D-600 and washed after 90 s with drug-free solution. Note that the negative inotropic effect was still increasing although the drug in the bath fluid had been removed. (b) Concentration-response relationship of D-600 in guinea-pig left auricles. $ED_{50}$ approx. $6 \times 10^{-8}$ M.

### *Effects of D-600 on force of contraction in guinea-pig left auricles*

Figure 5 shows the negative inotropic effect of D-600 in guinea-pig left auricles. $F_c$ was depressed in a concentration-dependent way. As with the optical isomers of D-600 in cat papillary muscles, the effects of the racemic mixture of D-600 in guinea-pig left auricles were not reversible at all concentrations tested after a 60 min washing period in drug-free solution. Even when the muscles were exposed only for a short time to D-600, $F_c$ did not return to control values. This effect is depicted in Figure 5a. The irreversibility of effects essentially also holds true for the analogous compound verapamil; Figure 6 shows the effects of

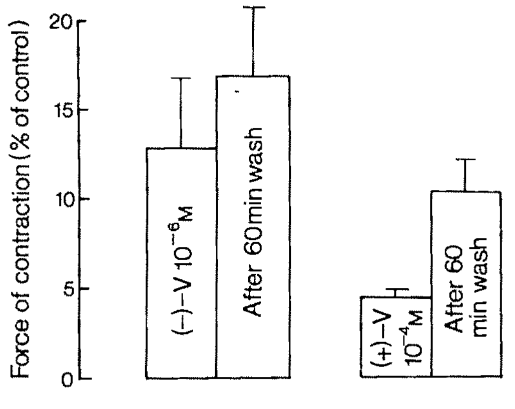

**Figure 6** Influence of (−)-verapamil $10^{-6}$ M and (+)-verapamil $10^{-4}$ M on $F_c$ in guinea-pig left auricles. Values are means of 4 preparations each. Vertical bars show s.e. mean. $F_c$ was greatly depressed and barely recovered after a 60 min washing period in drug-free solution.

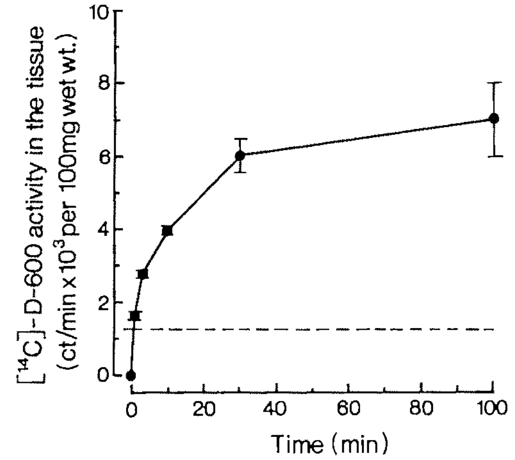

**Figure 7** Time course of the uptake of $10^{-6}$ M D-600-[nitrile-$^{14}$C] in guinea-pig left auricles. Values are means of 8–16 preparations each. Vertical bars show s.e. means. The activity of $10^{-6}$ M D-600-[nitrile-$^{14}$C] per 0.1 ml is indicated by the dotted line. Tissue-medium ratios ranged from 1.25 to 6.0.

(−)-verapamil $10^{-6}$ M and (+)-verapamil $10^{-4}$ M in guinea-pig left auricles. $F_c$ was greatly depressed at these concentrations and recovered only slightly after a 60 min washing period in drug-free solution.

Since the effects of (−)-D-600 are 100 times stronger than the effects of (+)-D-600, 99% of the action of the racemic mixture of D-600 on $F_c$ is quantitatively explained by the presence of (−)-D-600. In the racemic mixture of D-600, 50% of either optical form is to be expected; therefore, the response to D-600 is about half in comparison to that of (−)-D-600. This is in line with the $ED_{50}$ of about $3 \times 10^{-8}$ M for (−)-D-600 in cat papillary muscles (Figure 2) and of about $6 \times 10^{-8}$ M for D-600 in guinea-pig left auricles (Figure 5).

### *Uptake and release of D-600-[nitrile-$^{14}$C] in guinea-pig left auricles*

Since the effects of D-600 and its optical isomers were irreversible at all concentrations tested either in cat papillary muscles or in guinea-pig left auricles, a series of experiments was undertaken with D-600-[nitrile-$^{14}$C] to follow the uptake and release of the drug in the myocardial tissue. Auricles were taken in preference to papillary muscles because the specific activity of D-600-[nitrile-$^{14}$C] was not higher than 6.6 mCi/mM and effective doses were easier amd more precisely detected as radioactivity in tissue samples with greater weight: the mean wet weights ± s.d. amounted to 1.89 ± 1.03 mg in cat papillary muscles ($n = 107$) and to 28.9 ± 10.4 mg in guinea-pig left auricles ($n = 116$). Uptake measurements were made by immersing the preparations for 1 to 100 min in Tyrode solution containing $10^{-6}$ M D-600-[nitrile-$^{14}$C]. The time course of the uptake of the drug into the myocardial tissue is depicted in Figure 7. The concentration of the drug in the tissue was already about as high as in the Tyrode solution after 1 minute. From then on, the drug was accumulated to a value of about 6 times greater than the concentration in the Tyrode solution; equilibrium was reached after about 30 minutes. The time course of the uptake of D-600-[nitrile-$^{14}$C] seems to be reasonably related to the development of the negative inotropy as shown in Figure 8.

Figure 9 shows the release of the drug when the preparations were first loaded with D-600-[nitrile-$^{14}$C] for 100 min and then washed in drug-free solution for up to 5 hours. The accumulation of the drug was reversible after washing, indicated by the loss of radioactivity increasing with longer washing times. However, even after 5 h washout of the drug, a residual amount of radioactivity corresponding almost to a concentration of $10^{-6}$ M was found in the tissue. The release of D-600-[nitrile-$^{14}$C] does not seem to correspond to the total irreversibility of the negative inotropic effects after washing; on the other hand, the final amount of radioactivity found after long washing periods is reasonably high and might well be in line with the persistence of the negative inotropy once the effect has developed.

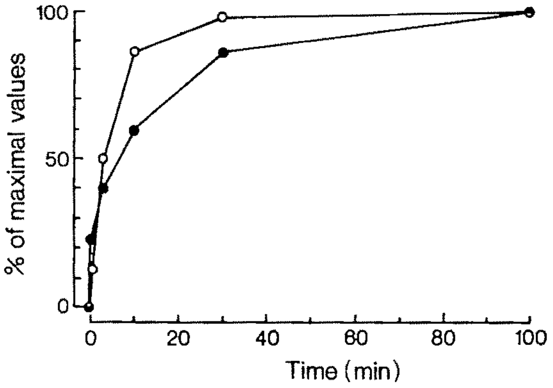

**Figure 8** Time course of the uptake of $10^{-8}$ M D-600-[nitrile-$^{14}$C] and development of the negative inotropy (same preparations as in Figure 7). Evaluation and expression of results are as described in Figure 3. $F_c$ was depressed to about 16% of control values after 100 minutes. Standard errors are omitted, since neither a statistically defined similarity would prove nor a dissimilarity would disprove a causal relationship between two completely different parameters. (O) Δ Force of contraction; (●) [$^{14}$C]-D-600 activity in the tissue.

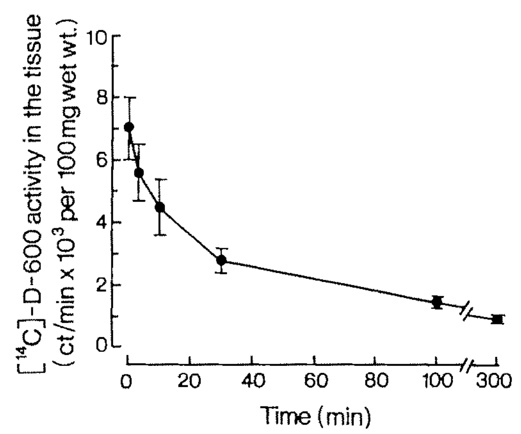

**Figure 9** Release of D-600-[nitrile-$^{14}$C] in guinea-pig left auricles which were first loaded with D-600-[nitrile-$^{14}$C] for 100 min and then washed for different time periods in drug-free solution. Values are means of 8–16 preparations each. Vertical bars show s.e. means. Although considerable amounts of the drug were being washed out, a residual radioactivity corresponding to almost $10^{-8}$ M was still found after 5 h of washing.

## Discussion

The results confirm the finding that D-600 and its optical isomers exert a concentration-dependent negative inotropic effect in the mammalian myocardium (Fleckenstein *et al.*, 1969; Bayer, Kaufmann & Mannhold, 1975). There were no differences found in the action of the optical isomers except in potency. This also applies to the influence of these drugs on the staircase phenomenon which was reduced at low and abolished at high concentrations. The latter finding corresponds to the data of McCans *et al.* (1974) who demonstrated that the negative inotropic effect of verapamil was also seen at all frequencies, but was more pronounced at higher frequencies of stimulation. The negative inotropic effect of D-600 can be explained by a decrease in the slow inward calcium current (Kohlhardt *et al.*, 1972) that is brought about by a decrease in the calcium conductance, and, at longer exposure times, also by an increase in the activation time of the calcium current (Nawrath, TenEick, McDonald & Trautwein, 1976).

Why the staircase phenomenon is diminished or abolished in the presence of these drugs is not known. Bayer *et al.* (1975) proposed, on the basis of computer calculations, that the cycling time of calcium in the sarcoplasmic reticulum might be prolonged by verapamil and D-600, thereby explaining the different response at different frequencies. It was claimed by the same authors that the staircase phenomenon was not only abolished but even reversed when the (—)-forms of either verapamil or D-600 were present. In the study of Bayer *et al.* (1975) $F_c$ was measured in isotonically contracting cat papillary muscles and $F_c$ was increased by about 20% when comparing 6 beats/min to 60 beats/minute. In our experimental set up, $F_c$ was measured in isometrically contracting cat papillary muscles and was increased by about 150% when comparing the same frequency difference. It might well be that under conditions where the staircase phenomenon is weak, a negative staircase can be established by verapamil or D-600. This also applies to the investigation of McCans *et al.* (1974) who described a very slight inversion of the positive staircase at higher concentrations of verapamil.

Furthermore, Bayer *et al.* (1975) stated that the positive staircase phenomenon was only affected by the (—)-isomers of verapamil and D-600 and virtually unaffected by the (+)-isomers. However, this conclusion was based upon the comparison of concentrations having quantitatively different effects on $F_c$.

Uptake measurements of D-600-[nitrile-$^{14}$C] in guinea-pig left auricles showed the drug to be accumulated in the myocardial tissue. Preparations which were first loaded with D-600-[nitrile-$^{14}$C] for 100 min and then washed for different times in drug-free solution lost up to about 80% of the radioactivity whereas the depression of $F_c$ remained virtually unchanged. The time course of the uptake of D-600-[nitrile-$^{14}$C] was similar to the development of the negative intropy. This suggests that the negative inotropic effect is somehow related to the binding and/or accumulation of D-600 in the myocardium. Which binding sites in the myocardial cell are important in mediating the negative inotropic effect remains an open question. The fact that D-600 accumulation is dependent on exposure time indicates the difficulty in obtaining exact concentration-response relationships. It seems only possible to compare the effects of defined bath concentrations in individual preparations at defined intervals after addition of the drug. Even then, when comparing different derivatives, a similar uptake behaviour has to be assumed.

Quite a reasonable amount of D-600 remained in the myocardium after exposure to the drug. If one assumes different binding sites for D-600 more or less determining the magnitude of the negative inotropy, this tightly bound D-600 is possibly responsible for the development and/or persistence of the negative inotropy. On the other hand, the possibility has not been excluded that the negative inotropy would persist without the D-600 that was still found in the tissue after 5 h washing.

In summary, it has been shown that (−)-D-600 and (+)-D-600 exert concentration-dependent negative inotropic effects with virtually no change in time to peak force or relaxation time. The negative inotropic effect of the racemic mixture of both isomers is mostly determined by the presence of (−)-D-600. Since there are only quantitative differences between the action of (−)-D-600 and (+)-D-600, there is no obvious advantage in using either of these isomers in preference to the racemic mixture. When D-600 is used in any studies of myocardial contractility the following limitations based on this study should be kept in mind: the effect of the drug on $F_c$ is not reversible at any concentration and the effect of the concentration investigated depends on the exposure time, since the drug is being accumulated in the myocardial tissue.

We thank Dr Thomas Meinertz for helpful discussions and suggestions. This investigation was supported by a grant from the Deutsche Forschungsgemeinschaft. The generous supply of D-600 and verapamil by Dr Oberdorf/Knoll AG is gratefully acknowledged.

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# EFFECT OF NEUROLEPTICS AND OTHER DRUGS ON MONOAMINE UPTAKE BY MEMBRANES OF ADRENAL CHROMAFFIN GRANULES

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1 The effects have been investigated of various reserpine-like, neuroleptic, antidepressant and other compounds on the adenosine-5′-triphosphate (ATP)-dependent uptake of noradrenaline (NA) (reserpine-sensitive) and tryptamine (reserpine-resistant) by membranes of isolated chromaffin granules of bovine adrenal medulla.

2 Reserpine and Ro 4-1284 (2-hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy-hexahydro-11bH-benzo(a)quinolizine) as well as neuroleptics (e.g. chlorpromazine and haloperidol) inhibited the NA uptake, but the reserpine-like drugs were more potent. In contrast, Ro 4-1284 showed a considerably weaker effect than the neuroleptics in interfering with tryptamine uptake. Chlorpromazine had about the same potency in inhibiting NA and tryptamine uptake, whereas the action of haloperidol was more pronounced on the uptake of NA than of tryptamine.

3 The relative potencies of neuroleptic drugs in inhibiting NA uptake by granule membranes *in vitro* corresponded only partly to their relative potencies in enhancing dopamine turnover *in vivo*.

4 The inhibition of NA uptake by chlorpromazine and Ro 4-1284 appeared to be of the non-competitive type.

5 Chlorpromazine did not influence the decrease in ATP induced by granule membranes in the incubation medium.

6 Other basic, but not acidic compounds also inhibited NA uptake by granule membranes; their potency was of the order of that of chlorpromazine (antidepressants) or weaker (e.g. benzodiazepines).

7 In conclusion, the mechanism of action of neuroleptics probably differs from that of reserpine-like drugs in the inhibition of monoamine uptake by membranes of catecholamine storage organelles. While interference with the granular storage of dopamine at the granule membrane level may contribute to the *in vivo* action of neuroleptics (e.g. in enhancing dopamine turnover), additional effects of these drugs must be involved *in vivo*, e.g. blockade of pre- and postsynaptic dopamine receptors.

## Introduction

Neuroleptic drugs of various chemical classes cause an increase of cerebral dopamine turnover (Pletscher & Kyburz, 1976). Several possible primary mechanisms leading to this functional change have been proposed. There is evidence for the presence of dopamine receptors at both pre- and postsynaptic sites, and inhibition of these receptors by the drugs probably leads to a feedback activation of neuronal dopamine turnover (Carlsson, 1975a). In addition, it has been suggested that neuroleptics affect presynaptic amine storage organelles (Seeman, 1972), although to date no direct proof for this hypothesis exists.

The present experiments were carried out in order to investigate the effects of neuroleptic and other drugs on the transport of labelled monoamines ([$^{14}$C]-noradrenaline and [$^{14}$C]-tryptamine) at the level of the membrane of amine storage organelles. The isolated membranes of adrenal chromaffin granules were used as a model system since it has not yet been possible to obtain pure amine storage organelles from the brain. Chromaffin granules are available in a highly purified form, and their membranes have been shown to take up biogenic amines by a specific, probably carrier-mediated adenosine-5′-triphosphate (ATP)-dependent transport mechanism (Taugner & Hasselbach, 1966; Taugner, 1971; Kirshner, 1974; Da Prada, Obrist & Pletscher, 1975). In the present investigations, [$^{14}$C]-noradrenaline ([$^{14}$C]-NA) was used instead of [$^{14}$C]-dopamine because the adrenal granule membranes contain dopamine-$\beta$-hydroxylase which transforms part of the [$^{14}$C]-dopamine into [$^{14}$C]-NA during the uptake process, whereas [$^{14}$C]-NA is taken up unchanged (Helle, 1971; Da Prada *et al.*, 1975). Previous (Da Prada *et al.*, 1975) as well as preliminary experiments showed that [$^{14}$C]-NA and [$^{14}$C]-dopamine were taken up to about the same extent by the granule membranes and that neuroleptic drugs affected the uptake of both amines similarly.

## Methods

### *Preparation of membranes*

Chromaffin granules were isolated from bovine adrenal medulla, lysed by hypotonic shock, isolated by ultracentrifugation, washed, dialysed for 20 min and resuspended in Na-glycerophosphate buffer pH 7.4 as previously described (Da Prada *et al.*, 1975). The final suspension contained about 800 μg protein per ml.

### *Equilibrium dialysis*

Equilibrium dialysis experiments were carried out at 37°C using a 'Dianorm' equilibrium dialyser (Diachema AG, Birmensdorf/Zurich, Switzerland) made of Teflon and containing two microchambers ($M_1$ and $M_2$) separated by a 'spectrapor 2TM' membrane (molecular weight cut-off: 12,000–14,000) (Spectrum Medical Industries Inc., Los Angeles, U.S.A.). One chamber contained the granule membranes, while the radioactive amines and the drugs were added to both chambers in equal initial concentrations. The difference in counts between chamber $M_1$ (with membranes) and chamber $M_2$ (without membranes) at the end of the incubation period was taken as a measure of the amine uptake by the granule membranes and expressed in nmol per mg protein (for details see Da Prada *et al.*, 1975). According to previous findings, the majority of [$^{14}$C]-NA probably accumulates in the interior of the membrane vesicles (newly formed from membrane fragments), whereas only minor amounts are bound to the membranes (Agostini & Taugner, 1973).

### *Assay methods*

Aliquots (40 μl) of the incubation mixture of both microchambers were placed in 10 ml of scintillation mixture (Aquasol, New England Nuclear, Boston, U.S.A.) and counted in an Isokap/300 liquid scintillation system (Nuclear Chicago, U.S.A.). Protein determination was carried out by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951). The ATP content in the microchambers was measured by the luciferin-luciferase method (Holmsen, Holmsen & Bernhardsen, 1966) before and during incubation for 90 minutes.

### *Materials*

Adenosine-5′-triphosphate disodium salt, Sigma grade, was obtained from Sigma, St. Louis, Mo, U.S.A., (−)-noradrenaline-[carbinol-$^{14}$C]-(±)-bitartrate (54 mCi/mmol) and tryptamine [1-$^{14}$C]-bisuccinate (60 mCi/mmol) from New England Nuclear, Boston, U.S.A. The source of the non-labelled compounds examined as inhibitors of amine uptake was as follows: 2-hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy-hexahydro-11bH-benzo(a)quinolizine (Ro 04-1284), chlordiazepoxide, chlorpromazine, diazepam, amitriptyline, chlorprothixene and methiotepin (F. Hoffmann-La Roche & Co. Ltd, Basel, Switzerland), imipramine, chlorimipramine and desmethylimipramine (Ciba-Geigy Ltd, Basel, Switzerland), thioridazine (Sandoz Ltd, Basel, Switzerland), clozapine (Wander Ltd, Bern, Switzerland), dextrobutaclamol and levobutaclamol (Ayerst Research Laboratories, Montreal, Canada), reserpine and phenobarbitone (Siegfried Ltd, Zofingen, Switzerland), γ-hydroxybutyrate (Aldrich Chemical Comp. Inc., Milwaukee, Wisc., U.S.A.), γ-aminobutyric acid (Serva Feinbiochemica, Basel, Switzerland), haloperidol and pimozide (Janssen, Beerse, Belgium), azure A (Aldrich, Beerse, Belgium) and fluorescein (Merck, Darmstadt, Germany).

## Results

### *Neuroleptics and reserpine-like drugs*

Chlorpromazine, haloperidol and the reserpine-like compound Ro 04-1284 (Pletscher, Brossi & Gey, 1962) inhibited the ATP-dependent uptake of NA by chromaffin granule membranes. The inhibition increased with increasing concentrations of the drugs and appeared to be of the non-competitive type (Figures 1 and 2). Various other neuroleptic drugs as well as reserpine decreased the NA uptake. Reserpine and Ro 04-1284 were between 30 and 2000 times more potent than the neuroleptics. Among the latter, haloperidol was the most potent compound exhibiting an $EC_{50}$ of $5 \times 10^{-6}$ M while the least potent drug, clozapine, differed by more than an order of magnitude. The $EC_{50}$ of L-butaclamol was only twice as high as that of D-butaclamol (Table 1).

### *Antidepressants*

These drugs also caused a concentration-dependent inhibition of the NA uptake by granule membranes. The compounds did not vary markedly in their potency which was of the same order as that of chlorpromazine and thioridazine, and thus lower than that of haloperidol (Table 1).

### *Miscellaneous compounds*

Other basic substances, e.g. benzodiazepines and the colourant Azure A, also interfered with the NA uptake in a concentration-dependent manner. Their potency, however, was rather weak, i.e. in the order of or below that of clozapine. In contrast, with the acidic compounds γ-hydroxybutyric acid, fluorescein, phenobarbitone as well as with γ-aminobutyric acid (GABA) little inhibition of NA uptake by granule membranes was detectable (Table 1).

### *Reserpine-resistant uptake*

In contrast to reserpine which did not inhibit the uptake of tryptamine (Da Prada *et al.*, 1975), chlorpromazine and haloperidol interfered with the uptake of this amine in a concentration-dependent manner. The $EC_{50}$ of chlorpromazine for inhibition of $[^{14}C]$-tryptamine uptake (about $4 \times 10^{-5}$ M) was similar to that for inhibition of $[^{14}C]$-NA uptake ($4 \times 10^{-5}$ M), whereas haloperidol was less potent in inhibiting tryptamine than NA uptake ($EC_{50}$ of haloperidol $7 \times 10^{-5}$ M and $5 \times 10^{-6}$ M, respectively). The reserpine-like compound Ro 04-1284 also interfered with tryptamine uptake, but its $EC_{50}$ ($8 \times 10^{-4}$ M) was considerably higher than that for inhibition of NA uptake ($1.5 \times 10^{-7}$ M) (Figure 3).

**Table 1** Molar concentration of various compounds causing a 50% inhibition of the (−)-$[^{14}C]$-noradrenaline uptake ($EC_{50}$) by membranes of adrenal chromaffin granules.

| *Drug* | *$EC_{50}$* |
|---|---|
| *Reserpine-like* | |
| Reserpine | $4 \times 10^{-8}$ |
| Ro 04-1284 | $1.5 \times 10^{-7}$ |
| *Neuroleptics* | |
| Haloperidol | $5 \times 10^{-6}$ |
| Pimozide | $1 \times 10^{-5}$ |
| D-Butaclamol | $2 \times 10^{-5}$ |
| Chlorprothixene | $3 \times 10^{-5}$ |
| Thioridazine | $3 \times 10^{-5}$ |
| Chlorpromazine | $4 \times 10^{-5}$ |
| L-Butaclamol | $4 \times 10^{-5}$ |
| Methiotepin | $5 \times 10^{-5}$ |
| Clozapine | $7 \times 10^{-5}$ |
| *Antidepressants* | |
| Chlorimipramine | $3 \times 10^{-5}$ |
| Imipramine | $4 \times 10^{-5}$ |
| Desimipramine | $4 \times 10^{-5}$ |
| Amitriptyline | $4 \times 10^{-5}$ |
| *Miscellaneous* | |
| Diazepam | $7 \times 10^{-5}$ |
| Azure A | $9 \times 10^{-5}$ |
| Chlordiazepoxide | $5 \times 10^{-4}$ |
| Phenobarbitone | $>5 \times 10^{-4}$ |
| γ-Aminobutyric acid | $>3 \times 10^{-3}$ |
| Fluorescein | $>5 \times 10^{-4}$ |
| γ-Hydroxybutyrate | $>3 \times 10^{-3}$ |

Incubation was at 37°C for 30 min in the presence of ATP. Initial concentrations of ATP and $[^{14}C]$-NA were 5 mM and 50 μM respectively. The values were determined graphically from concentration-inhibition curves (see Figures 1 and 3).

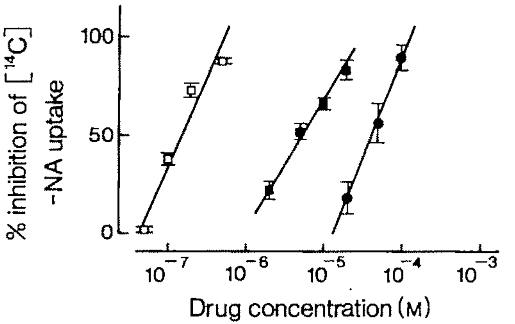

**Figure 1** Inhibition of the uptake of (−)-$[^{14}C]$-noradrenaline ($[^{14}C]$-NA) in membranes of adrenal chromaffin granules by Ro 04-1284 (☐), chlorpromazine (●) and haloperidol (■). Incubation at 37°C for 30 min in the presence of ATP. Initial concentrations of ATP and $[^{14}C]$-NA were 5 mM and 50 μM respectively. Each point is an average of 3 experiments. Vertical lines show s.e. mean.

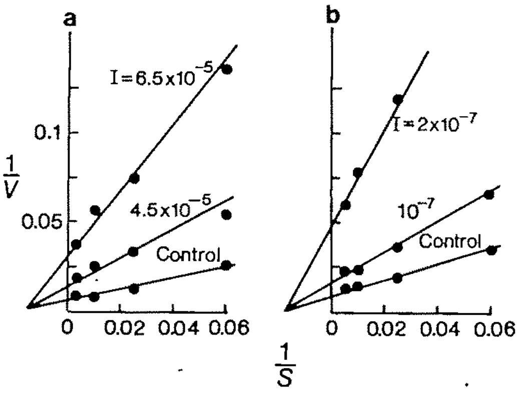

**Figure 2** Double reciprocal plot (Lineweaver-Burk) of the effect of (a) chlorpromazine and (b) Ro 04-1284 on the uptake of (−)-$[^{14}C]$-noradrenaline ($[^{14}C]$-NA) by membranes of adrenal chromaffin granules incubated for 30 min at 37°C in the presence of ATP (initial concentration 5 mM). $V$=initial velocity of uptake (nmol per mg of protein/15 min); $S$=μmolar concentration of $[^{14}C]$-NA. I=molar concentration of drugs. Typical experiments.

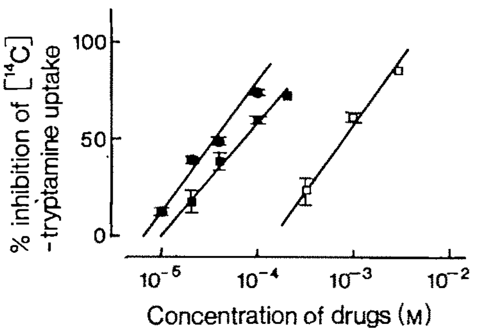

**Figure 3** Inhibition of the uptake of $[^{14}C]$-tryptamine by membranes of adrenal chromaffin granules caused by various concentrations of Ro 04-1284 (□), chlorpromazine (●) and haloperidol (■). Incubation at 37°C for 30 min in the presence of ATP. Initial concentrations of ATP and $[^{14}C]$-tryptamine were 5 mM and 50 μM respectively. Each point is an average of 3 experiments. Vertical lines show s.e. mean.

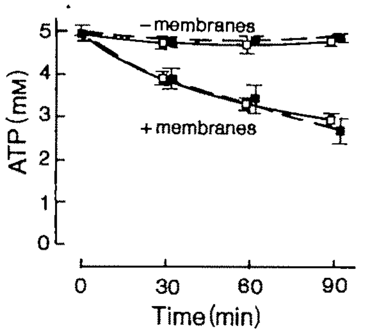

**Figure 4** Effect of chlorpromazine on the concentration of adenosine-5′-triphosphate (ATP) in the medium during incubation at 37°C with or without membranes of chromaffin granules. Each point is an average of 4 measurements performed in 2 experiments. Vertical lines show s.e. mean. Solid line: no chlorpromazine; broken line: with $10^{-4}$ M chlorpromazine.

### *ATP degradation*

Figure 4 shows that in the absence of membranes the ATP content of the medium remained unaltered for at least 90 minutes. However, in the presence of chromaffin granule membranes in one chamber there was a progressive decrease of the ATP content in both chambers and, as reported previously (Da Prada *et al.*, 1975), an appearance of adenosine-5′-diphosphate and adenosine-5′-monophosphate. Chlorpromazine ($10^{-4}$M) did not influence the ATP decrease induced by granule membranes (Figure 4).

## Discussion

The present results demonstrate that not only reserpine-like drugs (Taugner & Hasselbach, 1966; Da Prada *et al.*, 1975) but also neuroleptics inhibit the amine uptake by membranes of amine storage organelles. However, the mechanism of action of these two classes of drugs seems to differ from that of false neurotransmitters like octopamine. In fact, the antagonism between NA and octopamine was competitive (Da Prada *et al.*, 1975), while that between NA and Ro 04-1284 or chlorpromazine appeared to be of the non-competitive type. On the other hand, there was also a difference between neuroleptics and reserpine-like drugs. Neuroleptics, in addition to inhibiting the reserpine-sensitive NA uptake, also showed a relatively strong interference with the reserpine-resistant tryptamine uptake (compared to their effect on NA uptake), whereas reserpine-like drugs had a much greater effect on the reserpine-sensitive uptake (see Figures 1 and 3 and Da Prada *et al.*, 1975). These findings are compatible with the view that neuroleptics, such as chlorpromazine, cause an alteration of the physicochemical properties (e.g. fluidization) of artificial as well as biological membranes (e.g. of erythrocytes) (Seeman, 1972). This probably leads to relatively unspecific impairment of the monoamine transport system, whereas the more potent reserpine-like drugs might act through a more specific mechanism. It does not seem that chlorpromazine exerts its action by inhibiting membrane ATP-ase, since the drug, in concentrations causing a virtually complete block of monoamine uptake, did not greatly influence the ATP decrease in the medium induced by granule membranes. On the other hand, it has been suggested that reserpine interferes specifically with a transport ATP-ase (Taugner & Hasselbach, 1966), but no proof for this hypothesis exists as yet.

There might be some differences in the mode of action of chlorpromazine and haloperidol, since chlorpromazine affected NA and tryptamine uptake to the same extent, while haloperidol was more potent regarding interference with NA uptake.

The question arises whether the inhibition of amine uptake by reserpine-like and neuroleptic drugs seen in granule membranes *in vitro* is relevant for the *in vivo* action of the drugs. Both types of compounds enhance the dopamine turnover in brain *in vivo*.

Reserpine is thought to impair primarily the storage of amines in the presynaptic storage organelles (Carlsson, 1975b) leading to a virtually complete depletion of their dopamine content. The potent inhibitory effect of reserpine and Ro 4-1284 in the present as well as in previous experiments (Taugner & Hasselbach, 1966; Da Prada *et al.*, 1975) provides direct evidence that reserpine-like drugs exert their action at the level of the membrane of the storage organelles. As a consequence of the depletion of presynaptic dopamine, the neuronal turnover of the amine is enhanced, be it by removal of end-product inhibition of intraneuronal tyrosine hydroxylase or by disinhibition of pre- and postsynaptic dopamine receptors.

With regard to neuroleptics the relevance of the *in vitro* effect found in these experiments for their *in vivo* action is not fully clear. The action of these drugs on granule membranes was less potent than that of reserpine-like compounds. Furthermore, according to previous findings, the dopamine-sensitive adenylate cyclase in striatal homogenates (Iversen, 1975) which is considered to be related to the dopamine receptor (Kebabian, Petzold & Greengard, 1972), the electrically stimulated dopamine release from striatal slices (Seeman & Lee, 1975; Iversen, Horn & Miller, 1975), the presynaptic action of apomorphine on dopamine formation in striatal synaptosomes (Iversen, Rogawski & Miller, 1976) and the binding of haloperidol to postsynaptic receptors (Creese, Burt & Snyder, 1976) showed greater sensitivity to neuroleptics (one, to more than two, orders of magnitude) than the NA uptake by adrenal granule membranes. These findings might argue against neuroleptics exerting an effect *in vivo* by interfering with granular amine storage, but this argument is not fully convincing. Isolated membranes of adrenal chromaffin granules may be less sensitive to neuroleptics than brain monoamine storage organelles *in situ*, in fact, in brain slices the effective concentration of reserpine (which acts primarily on monoamine storage organelles) in enhancing spontaneous release of [$^{14}$C]-dopamine was of the order of $10^{-9}$ M (Seeman & Lee, 1974), whereas in the present experiments this order was $10^{-8}$ M. Also, the daily dose of neuroleptics, especially in psychiatry, is higher than that of reserpine (e.g. 200 mg chlorpromazine or approximately $10^{-5}$ M/kg) and furthermore neuroleptics are administered for a prolonged period of time, whereas the present work deals with acute experiments. An enhanced release of dopamine (which might be due to an impairment of amine storage) has, for instance, been suggested as the cause of some side effects of neuroleptic drugs, e.g. the tardive dyskinesia (Seeman & Lee, 1974).

An action of neuroleptics on granular amine storage may also contribute to an increased turnover of dopamine *in vivo*, although neuroleptics, in contrast to reserpine-like drugs, do not cause a depletion of presynaptic dopamine stores (probably owing to a compensatory increase of dopamine synthesis). It has been suggested that an increased release of dopamine due to the action of neuroleptics on dopamine stores removes end-product inhibition of tyrosine hydroxylase, thereby enhancing dopamine synthesis (Seeman, 1972).

While an action of neuroleptic drugs on the membranes of dopamine storage organelles may be a contributory element, other factors must be involved in the *in vivo* action of neuroleptic drugs. Thus, D-butaclamol enhanced the turnover of dopamine *in vivo* much more than the L-enantiomer (which was virtually inactive) (Lippmann, Puglsey & Merker, 1975), whereas in the NA uptake experiments with granule membranes L-butaclamol was only slightly inferior to the D-form. Furthermore, clozapine *in vivo* was at least one order of magnitude less potent than chlorpromazine (Pletscher, 1975), whereas *in vitro* the difference was smaller. Finally, antidepressant drugs and benzodiazepines which differ from neuroleptics in their clinical spectrum as well as in their effect on cerebral dopamine turnover (Da Prada & Pletscher, 1966; Bartholini, Keller, Pieri & Pletscher, 1973), also showed some inhibitory effect on NA transport in granule membranes. These findings are probably due to the fact that additional mechanisms are involved in the *in vivo* action of the various neuroleptics, e.g. stimulation of pre- and postsynaptic dopamine receptors, inhibition of $Ca^{2+}$ influx in presynaptic nerve terminals leading to a blockade between nerve impulse and neurosecretion (Seeman & Lee, 1975). Furthermore, the action of drugs on granular membranes may be masked by an interference of the drugs with other processes, e.g. amine uptake at the neuronal membrane (antidepressants (Halaris, Belendiuk & Freedman, 1975)) or with other transmitters, e.g. GABA (benzodiazepines (Keller, Schaffner & Haefely, 1976)). Finally, *in situ*, the various neuroleptics and other drugs are possibly transported across the neuronal membrane to different degrees leading to differences in the intraneuronal drug concentration.

It is of interest that different types of basic substances interfered with the amine transport in granule membranes, whereas acidic compounds did not cause a relevant inhibition of the NA uptake (Table 1). These results are in agreement with previous findings in blood platelets where various basic compounds, especially neuroleptics, reserpine-like substances and colourants (but according to preliminary results not acidic dyes) showed a rather selective localization at the level of the 5-hydroxytryptamine storage organelles (Da Prada & Pletscher, 1969; 1975).

Some preliminary results of this work were presented at the Symposium on Physiology and Pathology of Biological Membrane Functions organized by the Swiss Academy of Medical Sciences in the spring of 1976.

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*(Received August 23, 1976.)*

*Br. J. Pharmac.* (1977), 59, 425–432

# INHIBITION BY GLUCOCORTICOIDS OF PROSTAGLANDIN RELEASE FROM ADIPOSE TISSUE *in vitro*

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1 When rabbit chopped adipose tissue was incubated with a lipolytic agent (adrenocorticotrophic hormone, ACTH$^{1-24}$, 0.1 μg/ml) in Krebs solution, prostaglandin $E_2$ was formed in the tissue and about the same amount was found in the medium.

2 In the presence of indomethacin (1 μg/ml) the appearance of prostaglandin $E_2$ was almost abolished both in the tissue and in the medium.

3 When the incubation was carried out in the presence of hydrocortisone or betamethasone (1–10 μg/ml) the concentration of prostaglandin $E_2$ leaking or carried into the medium was significantly reduced, whereas that remaining in the tissue was significantly increased. This action of the steroids was not reversed by increasing substrate (arachidonic acid) concentration in the medium.

4 The steroids did not affect lipolysis, nor did they influence prostaglandin metabolism since such activity was not detectable in the adipose tissue.

5 Anti-inflammatory steroids therefore did not reduce prostaglandin formation but increased the tissue/medium ratio, which supports the view that they inhibit the release of prostaglandins after these have been synthesized.

## Introduction

The functional vasodilatation which accompanies lipolysis in rabbit subcutaneous adipose tissue is mediated by a prostaglandin which is probably prostaglandin $E_2$ (Lewis & Matthews, 1970; Bowery, Lewis & Matthews, 1970). During lipolysis it was possible to detect prostaglandin formation within the fat tissue and its identification as prostaglandin $E_2$ has been confirmed by mass spectroscopic analysis (Dalton & Hope, 1974). Later, Lewis & Piper (1976) found that small amounts of a prostaglandin-like substance were released during lipolysis from the fat tissue into the venous blood. Following the discovery that non-steroid anti-inflammatory agents prevent prostaglandin formation (Vane, 1971), it was shown that such anti-inflammatory agents inhibit prostaglandin formation and functional vasodilatation in subcutaneous fat but do not prevent lipolysis (Bowery & Lewis, 1973). Therefore, it seems likely that inhibition of the vasodilatation was the result of inhibition of prostaglandin synthetase activity in adipose tissue.

Lewis & Piper (1975) have subsequently shown that like non-steroid anti-inflammatory drugs, anti-inflammatory steroids such as hydrocortisone, betamethasone and prednisolone inhibit the prostaglandin-mediated functional vasodilatation in subcutaneous adipose tissue. However, the mechanism of action of the steroids is different. Like indomethacin, hydrocortisone inhibits the vasodilatation and does not antagonize the direct vasodilator action of prostaglandins or reduce lipolysis. However, whereas indomethacin inhibits the formation of prostaglandin in the fat tissue itself, hydrocortisone does not.

We have therefore postulated that anti-inflammatory steroids inhibit the release of prostaglandins after they have been formed in the fat cells.

We have now confirmed the *in vivo* experiments by an *in vitro* study of the changes taking place during lipolysis in chopped fat. A preliminary account of this work was given to the British Pharmacological Society (Chang, Lewis & Piper, 1976).

## Methods

### *Preparation of chopped fat*

Female New Zealand white rabbits weighing 3.0 to 3.5 kg were killed by a blow on the head. The epigastric fat pads were dissected out, rinsed in Krebs bicarbonate solution and divided in 5 g portions. Each portion was chopped into pieces about 2 mm$^3$ and washed three times with fresh Krebs solution. The fat was then pre-incubated for 20 min at room temperature with or without the anti-inflammatory drug before lipolysis was induced by the addition of adrenocorticotrophic hormone$^{1-24}$ (ACTH). The final volume of the incubation medium was 10 ml and total incubation time was usually 2 hours.

### *Preparation of isolated fat cells*

Isolated fat cells were prepared from rabbit epigastric fat pads by the method of Rodbell (1964). The standard isolation and incubation medium was Krebs bicarbonate solution containing 2% bovine serum albumin. The medium was kept at pH 7.4 by bubbling with 95% $O_2$ and 5% $CO_2$. The two epigastric fat pads were pooled, cut into small pieces and 5 g was transferred to an incubation vial containing 10 mg of collagenase (Worthington Biochemicals) in 4 ml of the incubation medium. Cells were washed three times with 5 ml of the standard medium and finally suspended in this solution. All vessels used in the preparation were made of plastic or siliconized glass. Incubation of cells was carried out with 1 ml of the cell suspension. The dry weight of 1 ml of cells was determined by the difference between the weight of 1 ml of dried cell suspension and the weight of 1 ml of medium dried overnight at 70°C.

### *Glycerol assay*

Glycerol was assayed by the method of Eggstein & Kreutz (1966). Glycerol release was taken as the index of lipolysis since the rate of re-utilization of glycerol by fat cells is minimal owing to the lack of glycerolkinase (Margolis & Vaughan, 1962).

### *Prostaglandin assay and thin layer chromatography*

Immediately after incubation, fat was filtered from the incubation medium and an aliquot of ice-cold Krebs bicarbonate solution containing indomethacin (10 µg/ml) was added to the fat which was then filtered a second time. The two filtrates were combined. The chopped fat was then placed in ice-cold 4% ethanolic solution (brought to pH 3–3.5 with 1 N HCl) which also contained indomethacin. The tissue was homogenized and centrifuged at 10,000 *g* at 0°C. Prostaglandins were extracted from the supernatant with ethyl acetate at pH 3. The percentage recovery of prostaglandins from the incubation medium and fat tissue was monitored by adding 5,000 d/min per 100 µl of [$^3$H]-prostaglandin $E_2$ before extraction. Identification of the prostaglandin(s) released from the adipose tissue was carried out by thin layer chromatography using the AI and AII systems of Grēen & Samuelsson (1964).

The prostaglandin content of the tissue and supernatant (i.e. incubation medium) was measured by radioimmunoassay (Hennam, Johnson, Newton & Collins, 1974) with modifications (Jose, Niederhauser, Piper, Robinson & Smith, 1976). Tritiated prostaglandin $E_2$ (sp. act. 160 Ci/mmol; 20,000 d/min ≡ 20 pg) and an anti-serum to prostaglandin $E_2$ (diluted to bind 50–60% of [$^3$H]-prostaglandin $E_2$ in the absence of additional prostaglandin $E_2$) were used in the assay system. Ammonium sulphate solution was used to precipitate the antibody-bound material and this fraction was resuspended in distilled water, mixed with Instagel and the radioactivity determined.

The standard curve covers the range 0–1000 pg but only the linear part of the curve (60–400 pg) was used. The method blank was routinely determined by extraction of 10 ml of Krebs-albumin solution. The values were often lower than the linear range of the curve but corresponded approximately to 40–60 pg/ml.

The cross-reactions with other prostaglandins are as follows: prostaglandin $E_1$:43.8%; $F_{2\alpha}$ :1.3%; $F_{1\alpha}$:0.7% $A_2$:0·6%; $D_2$:0.1%; 15-keto-prostaglandin $E_2$:18.8%; 13, 14 dihydro-15-ketoprostaglandin $E_2$: 0.2%; 13, 14 dihydro prostaglandin $E_2$:14.5%. (Determined from mass required to give 50% displacement of [$^3$H]-prostaglandin $E_2$.) The estimates of prostaglandin content obtained by radioimmunoassay were checked by bioassay on superfused rat stomach strip, chick rectum and rat colon in the presence of suitable antagonists (Piper & Vane, 1969).

### *Crude preparation for examination of prostaglandin metabolism*

Fat tissue was rinsed in ice-cold buffer and homogenized in two volumes (w/v) of ice-cold 100 mM Tris buffer pH 8.0. The supernatant obtained from successive centrifugation at 10,000 *g* for 30 min and 100,000 *g* for 60 min was divided into 5 ml aliquots. The aliquots were stored at −20°C and kept no longer than a week. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

To 5 ml aliquots were added 2 µmol NAD and prostaglandin $E_2$ (100 ng). The mixtures were incubated for 2 h at 37°C and the reaction terminated by plunging the tubes into boiling water. Control samples of boiled enzyme with or without added prostaglandin $E_2$ were also extracted as described above and bioassayed on the superfused rat stomach strip together with the experimental samples.

### *Partitioning of [$^3$H]-prostaglandin $E_2$ in fat tissue*

The epigastric fat pads were removed from the rabbits and fragments of tissue not exceeding 200 mg were incubated in Krebs bicarbonate solution (pH 7.4) containing [$^3$H]-prostaglandin $E_2$. In all experiments the tissues were incubated for 2 h and the medium kept at 37°C. Sufficient [$^3$H]-prostaglandin $E_2$ was used in each experiment to give approximately 4,000 counts per minute (ct/min) in 100 µl. This amount of radioactivity was determined accurately each time by counting a 100 µl aliquot of the standard [$^3$H]-prostaglandin $E_2$ solution. At the end of the incubation period the tissue fragments were removed and blotted dry on filter paper. The tissues were then transferred to counting vials containing 2 ml of Soluene 350 (Packard) and left overnight for digestion. After digestion the sample was counted in 10 ml of Instagel. Previous experiments have shown that the samples were thoroughly quenched after 18 h and quenching in each sample was monitored by the external standard method.

The results are presented as the tissue to medium ratio (T/M). The ratio is calculated by the equation:

$$T/M = \frac{\text{ct/min per 100 mg tissue}}{\text{ct/min per 100 µl medium}}$$

*Incorporation of [$^{14}$C]-arachidonic acid into cell lipids*

In an initial experiment, a time course for incorporation of [$^{14}$C]-arachidonic acid was measured. It was found that incubation for 4 h was required for maximum incorporation. Thereafter the chopped fat was labelled for this period. Immediately following labelling, excess label was removed from the fat by several washings. The efficiency of removal of labelled material was checked by counting the radioactivity in aliquots of the fluid used for each wash; only 0.1–0.3% of the radioactivity remained after the fourth wash. After uptake of [$^{14}$C]-arachidonic acid the fat tissue was placed in fresh incubation medium containing ACTH (1 µg/ml) and incubated for 1 hour.

*Effect of steroid on ACTH-induced [$^{14}$C]-prostaglandin synthesis*

The fat was incubated in an incubation medium containing hydrocortisone (100 µg/ml) for 2 h and then [$^{14}$C]-arachidonic acid was added and incubation continued for 4 hours. Immediately following the uptake of [$^{14}$C]-arachidonic acid the fat was stimulated with ACTH in the presence of hydrocortisone. Control incubations of fat tissue received an equivalent volume of 0.9% w/v NaCl solution (saline).

In another set of experiments, 'cold' arachidonic acid complexed to bovine serum albumin was added to the medium after the uptake of [$^{14}$C]-arachidonic acid. The complex was formed in the following way. An aliquot of arachidonic acid in *n*-hexane was taken down to dryness. The dried sample was redissolved in 100 µl of ethanol and added to a solution of 1 g of albumin in 10 ml saline at 30°C. The solution took approximately 30 min to clear. This solution was then adjusted to give the required concentration of arachidonic acid. The final concentration of the arachidonic acid was 10 µg/ml. ACTH and hydrocortisone were added as described above. Levels of $^3$H or $^{14}$C in extracts were counted in a Tricarb liquid scintillation counter.

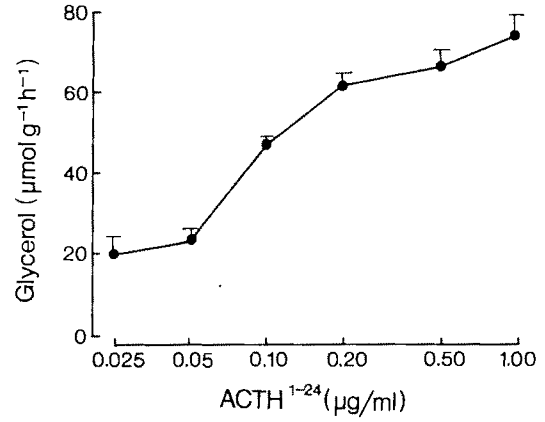

Figure 1 ACTH$^{1-24}$-induced lipolysis in chopped epigastric adipose tissue of rabbit. Results are µmol of glycerol formed per g of chopped fat in 1 h when incubated with increasing concentrations of ACTH$^{1-24}$. Vertical lines show s.e. means. Basal level = 10.24 ± 0.41 µmol g$^{-1}$ h$^{-1}$.

*Materials used*

Drugs used were: ACTH$^{1-24}$ (Synacthen, CIBA); bovine serum, albumin (Sigma); arachidonic acid (Sigma); [$^{14}$C]-arachidonic acid (58 mCi/mmol, Radiochemical Centre, Amersham); betamethasone sodium phosphate (Glaxo Laboratories); hydrocortisone 21-sodium succinate (Sigma); indomethacin (Merck, Sharpe & Dohme).

The Krebs bicarbonate solution had the following composition in g/l (mM): NaCl 6.9 (118), KCl 0.35 (4.7), $CaCl_2.6H_2O$ 0.55 (2.5), $KH_2PO_4$ 0.16 (1.2), $MgSO_4$ $7H_2O$ 0.29 (1.17), glucose 1.0 (5.6) and $NaHCO_3$ 2.1 (25.0).

## Results

*Lipolysis and prostaglandin formation*

When subcutaneous adipose tissue was taken from the epigastric region of rabbits, immediately chopped and incubated with ACTH for 2 h, lipolysis could be measured by estimation of glycerol release. Figure 1 shows the relationship between lipolysis and concentration of ACTH over a range of 0.01–1.0 µg/ml. It can be seen that ACTH 0.1 µg/ml produced about 50% of the maximum formation of glycerol. This con-

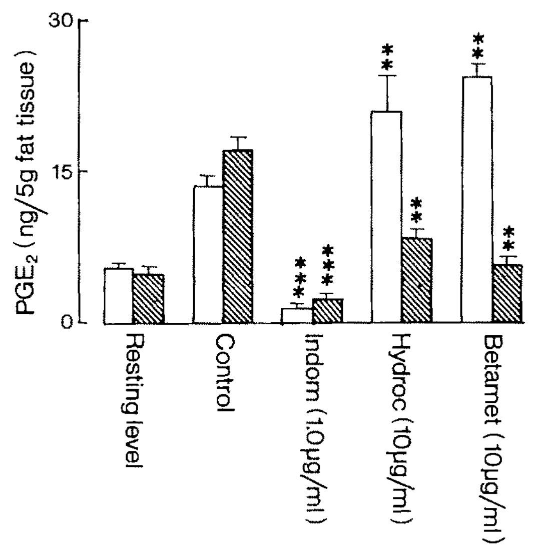

**Figure 2** Prostaglandin content (ng/5g tissue) measured by radioimmunoassay, in chopped fat tissue (open columns) and supernatant obtained after separation of the 2 phases (hatched columns). The resting level was obtained by incubating the chopped fat alone and the control level by incubating with 0.1 µg/ml ACTH$^{1-24}$ for 2 hours. The effect of anti-inflammatory drugs was examined by carrying out the incubations in the presence of indomethacin (Indom) 1.0 µg/ml, hydrocortisone (Hydroc) 10 µg/ml or betamethasone (Betamet) 10 µg/ml. Each column represents the mean of 4–6 experiments. Vertical lines show s.e. means. $^{**}P<0.01$; $^{***}P<0.001$.

centration was therefore used in subsequent experiments in which prostaglandin was estimated by radioimmunoassay during lipolysis. Incubation of chopped fat in Krebs solution alone resulted in a small formation in the fat tissue and supernatant of a prostaglandin-like material which co-chromatographed with prostaglandin $E_2$ in the AI and AII solvent systems. The concentration in the fat was 1.1 ± 0.4 ng/g and in the supernatant 1.0 ± 0.5 ng/ml.

On incubation with ACTH 0.1 µg/ml for 2 h there was a significant increase in the prostaglandin content of both the fat tissue (2.7 ± 0.7) ng/g and the supernatant (3.4 ± 1.3) (Figure 2). These results are somewhat lower than those reported earlier using ACTH 1 µg/ml (Bowery *et al.*, 1970) but the ratio of prostaglandin in resting to stimulated fat is of the same order. During ACTH-stimulated lipolysis prostaglandin synthesis also occurs in isolated fat cells. The amount of prostaglandin $E_2$ found after incubation with ACTH 0.1 µg/ml was 2–3 ng/g fat cells which accounted almost wholly for the prostaglandin formed in the chopped fat preparation.

### *Effect of anti-inflammatory agents*

In the presence of prostaglandin synthetase inhibitors represented here by indomethacin, or anti-inflammatory steroids such as hydrocortisone and betamethasone, ACTH 0.1 µg/ml continued to cause the release of glycerol, indicating that lipolysis still occurred as shown in Table 1.

However, when the effect of anti-inflammatory agents on prostaglandin in fat tissue and supernatant was examined the result was quite different. In the presence of indomethacin 1 µg/ml the prostaglandin content of both tissue and supernatant was reduced to a value which was even lower than that found after incubation with Krebs solution alone, as shown in Figure 2. On the other hand, when the incubation was carried out in the presence of hydrocortisone or betamethasone although the amount of prostaglandin released into the supernatant was reduced, the tissue prostaglandin content was significantly higher than that found after incubation with ACTH alone (Figure 2). This effect of the steroid was dose-dependent and Figure 3 shows the effect of betamethasone over a dose range of 1–10 µg/ml. Thus, whereas indomethacin reduced the overall amount of prostaglandin, the steroids altered the ratio of prostaglandin concentration in adipose tissue to prostaglandin concentration in the supernatant. The increase in the tissue/medium ratio of prostaglandin with increasing concentrations of hydrocortisone and betamethasone is illustrated in Figure 4.

**Table 1** Effects of anti-inflammatory drugs on the lipolytic action of ACTH$^{1-24}$

| *Drug* | (n) | *(µg/ml)* | *Glycerol levels following subsequent incubation with ACTH* |
|---|---|---|---|
| Control | (4) | — | 33.54 ± 1.05 |
| Indomethacin | (4) | 1.0 | 32.98 ± 0.6 (NS) |
| Hydrocortisone | (4) | 10.0 | 33.67 ± 0.73 (NS) |
| Betamethasone | (4) | 10.0 | 34.36 ± 0.55 (NS) |

Significance between control and test drug was calculated using Student's *t* test: NS = $P>0.05$.

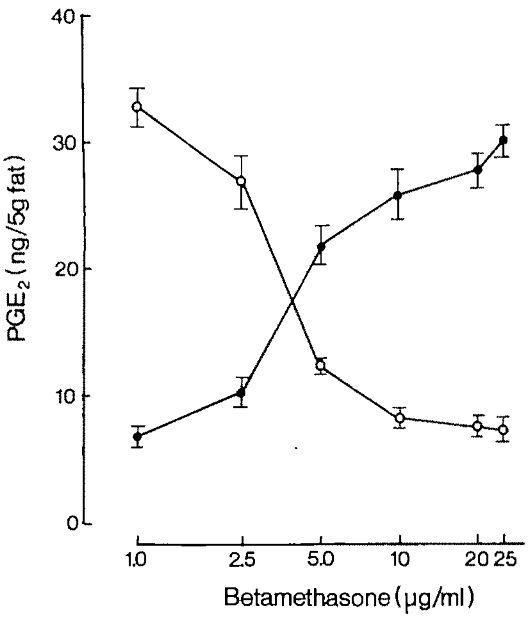

**Figure 3** Effect of betamethasone on the prostaglandin content of tissue (●) and supernatant (medium) (○). Incubations were carried out for 2 h in the presence of ACTH$^{1-24}$ 0.1 µg/ml and various concentrations of betamethasone. Prostaglandin concentration was estimated by radioimmunoassay. Each point is the mean of 4–6 experiments. Vertical lines show s.e. means.

## *Effect of arachidonic acid*

It has been suggested (Gryglewski, Bogumila, Korbut, Grodzinska & Ocetkiewicz, 1975) that steriods inhibit the appearance of prostaglandins in some tissues by interfering with the availability of the substrate precursor, arachidonic acid, and that addition of arachidonic acid reverses the effect of the steroid. When [$^{14}$C]-arachidonic acid (10 µg/ml) was incubated with chopped adipose tissue for up to 4 h there was a progressive uptake of 70–80% of [$^{14}$C]-arachidonate into the fat. Incubation of the adipose tissue with ACTH resulted in the release of [$^{14}$C]-prostaglandin-like material. The percentage conversion of [$^{14}$C]-arachidonic acid to prostaglandin-like material was 0.2–0.5%. When fat tissue was incubated with hydrocortisone or betamethasone prior to incubation with [$^{14}$C]-arachidonate and then stimulated with ACTH, the ratio of prostaglandin in the tissue to supernatant was increased as in the experiments without arachidonic acid. This ratio was the same in other experiments in which the substrate concentration was increased by the addition of arachidonic acid complexed to albumin.

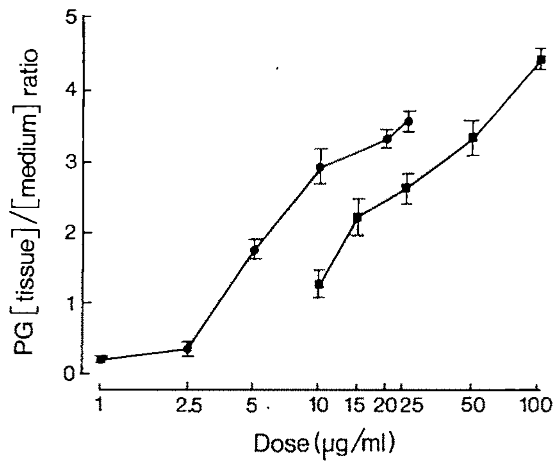

**Figure 4** Ratio of prostaglandin content of tissue/prostaglandin content of supernatant (medium). Incubations of chopped fat tissue were carried out in the presence of ACTH$^{1-24}$ 0.1 µg/ml and various concentrations of hydrocortisone (■) or betamethasone (●). Each point is the mean ratio of 4–6 experiments. Vertical lines show s.e. means.

## *Partitioning of prostaglandins*

A possible alternative to the hypothesis that steroids prevent the release of prostaglandins from fat cells could be that steroids influence the uptake of prostaglandin into the fat tissue. Table 2 shows that when fat tissue was incubated with Krebs solution containing [$^{3}$H]-prostaglandin $E_2$, about 30% was taken up into the tissue. This distribution of [$^{3}$H]-prostaglandin $E_2$, between tissue and fluid is in good

**Table 2** Proportion of [$^{3}$H]-prostaglandin $E_2$ taken up by the chopped fat preparation, expressed as tissue to medium ratio

| | *Tissue/Medium (T/M)* |
|---|---|
| Control (without ACTH) | 0.37 ± 0.02 |
| ACTH | 0.38 ± 0.03 (NS) |
| ACTH + hydrocortisone | 0.38 ± 0.03 (NS) |
| ACTH + betamethasone | 0.36 ± 0.03 (NS) |
| Betamethasone | 0.36 ± 0.05 (NS) |
| Hydrocortisone | 0.38 ± 0.06 (NS) |

T = counts per minute corrected for 100 mg fat tissue; M = counts per minute corrected for 100 µl medium.
Significance between control and test drug was calculated by Student's *t* test: NS = $P > 0.05$. All ratios are expressed as means ± s.e. mean; $n = 4$ in all cases.

agreement with the findings of Bito (1972) for fat tissue. When the incubation was carried out in the presence of hydrocortisone (10 µg/ml) or betamethasone (10 µg/ml) this distribution was unaltered.

### *Inactivation of prostaglandins*

When prostaglandin $E_2$ was incubated for up to 2 h with the supernatant obtained by centrifuging homogenized adipose tissue at 100,000 *g* no inactivation of prostaglandin $E_2$ could be detected. However, when prostaglandin $E_2$ was incubated with the high speed supernatant of guinea-pig or rabbit lung, prepared under the same conditions, metabolism and inactivation occurred as has been described by many workers.

## Discussion

The experiments described have shown that whereas a non-steroid anti-inflammatory agent, indomethacin, reduced the total amount of prostaglandin formed during lipolysis in chopped fat, anti-inflammatory steroids did not reduce the total formation but increased the tissue/medium ratio. In retrospect, further evidence of this inhibition of prostaglandin release by steroids was provided by Eakins, Whitelocke, Bennett & Martenet (1972). They detected the release of prostaglandin-like activity into the aqueous humour of patients with anterior uveitis. After local steroid treatment, prostaglandin-like activity was no longer found in the aqueous humour.

Since the discovery that non-steroid anti-inflammatory drugs produce at least some of their anti-inflammatory effects by inhibiting prostaglandin synthetase and therefore preventing the formation of prostaglandins, it has been difficult to explain similar anti-inflammatory activities of corticosteroids since it has been demonstrated by several groups of workers that anti-inflammatory steroids do not inhibit prostaglandin synthetase (Vane, 1971; Flower, Gryglewski, Herbaczynska-Cedro & Vane, 1972; Greaves, Kingston & Pretty, 1975; Lewis & Piper, 1975). In conditions where prostaglandin formation might occur extracellularly or where the cells are damaged so much that their membranes no longer provide a barrier to the diffusion of materials, prostaglandin synthetase inhibitors would be expected to prevent the appearance of prostaglandins whereas corticosteroids would not. Anti-inflammatory steroids did not prevent the release of prostaglandins from isolated strips of rabbit jejunum or perfused spleen, whereas prostaglandin synthetase inhibitors prevented their appearance (Lewis & Piper, 1975; Ferreira, Moncada & Vane, 1971). In both cases cells may have been altered or damaged; in the jejunum, prostaglandin formation was probably due to cell breakdown and in the spleen, cell membranes may have been altered by prolonged perfusion with Krebs solution and dextran. However, inhibition of prostaglandin release could well provide an explanation for the local vasoconstrictor activity of glucocorticoids in skin (McKenzie, 1962). During lipolysis there is no damage to the fat cells and release of prostaglandins from these cells is therefore probably a carrier-mediated process rather than leakage. As steroids do not alter the uptake of prostaglandins into the chopped adipose tissue, it seems to be this carrier-mediated release which is inhibited by these drugs. It is not yet known whether glucocorticoids act in other tissues as they appear to do in adipose tissue, i.e. inhibiting the release of prostaglandin, but there are some indications that this might be so.

Gryglewski *et al.* (1975) found that hydrocortisone and dexamethasone inhibited the release of prostaglandins from isolated blood vessels caused by the action of catecholamines, and from guinea-pig perfused lungs during anaphylaxis. These authors also reported that the steroid inhibition of prostaglandin release was reversed by the prostaglandin precursor, arachidonic acid, and concluded that the steroids prevented prostaglandin release by impairing the availability of substrate for prostaglandin synthetase. The uptake of arachidonate into lung tissue in such a way that it can be converted to prostaglandin is a slow process (Crutchley, Piper & Seale, unpublished observations). It seems unlikely that in the experiments of Gryglewski *et al.* (1975) the perfused arachidonic acid reached the site at which endogenous arachidonic acid is released during lipolysis. It might remain in or around the pulmonary vasculature where steroids do not inhibit the conversion of arachidonic acid to prostaglandins (Dawson & Tomlinson, 1974). Such a situation might explain the apparent removal of steroid inhibition by infusion of arachidonic acid. In experiments with rabbit adipose tissue *in vivo* (Lewis & Piper, unpublished observations) arachidonic acid failed to restore the prostaglandin-mediated vasodilatation during lipolysis after inhibition by steroids. In the present experiments arachidonic acid did not reverse the effect of the glucocorticoids *in vitro*, although the [$^{14}$C]-arachidonic acid administered was available for conversion into prostaglandins. We therefore conclude that, at least in adipose tissue, steroids do not prevent release of prostaglandins by blocking the availability of substrate to the enzyme but by some other mechanism.

The authors of two recent papers have concluded that anti-inflammatory steroids inhibit synthesis of prostaglandins rather than their release, but do not provide very firm evidence for specific inhibition of prostaglandin synthetase. In the first paper Kantrowitz, Robinson, McGuire & Levine (1976) found that relatively low concentrations of anti-inflammatory steroids appeared to inhibit prostaglandin synthesis in transplants of rheumatoid synovia. In this paper prostaglandins were identified and estimated by radioimmunoassay alone; this would have been more satisfactory if supported by evidence of chemical extraction or biological assay of prostaglandins, since difficulties are common particularly in the case of antiserum to prostaglandin $E_2$ and unsupported results often unreliable. It appears more likely that the steroids were producing some sort of toxic effect on the cells as suggested by the authors. Their findings from one experiment, based on a routine histological examination of haematoxylin and eosin-stained sections, that there were no apparent differences between control and dexamethasone-treated cells do not exclude a possible biochemical lesion in the transplants. Such a toxic effect might well lead to a non-specific inhibition of enzyme activity in the transplants.

In the second of these reports, Tashjian, Voelkel, McDonough & Levine (1976) examined the amount of prostaglandin formed during incubation of mouse fibrosarcoma cells. The experimental results support our working hypothesis and it is only the authors' interpretation which is in contention since they found that there was a dose-related inhibition of prostaglandin release into the incubation medium by low concentrations of hydrocortisone ($5 \times 10^{-9}$ to $5 \times 10^{-7}$M) while formation of prostaglandins in the cells continued and was not significantly different from control incubations without steroid. We conclude from their findings that the release of prostaglandins formed within the cells was inhibited by the corticosteroids since the tissue/medium ratio increased from 0.4 to 2.0 (rough calculation from data in Tashjian *et al.*, 1976). The authors' findings that there was no increase in intracellular prostaglandin in the presence of hydrocortisone cannot be regarded as evidence against this conclusion. Firstly, synthesis was not inhibited and secondly, no analysis was made to find why prostaglandin accumulation did not occur. For example, unlike rabbit fat cells, mouse fibrosarcoma cells might contain prostaglandin metabolizing enzymes that would tend to keep the intracellular concentration low.

We therefore conclude that in rabbit adipose tissue, and possibly also in other tissues, anti-inflammatory steroids prevent the release of prostaglandins, probably by interfering with a transport mechanism, but do not affect the synthesis of prostaglandins.

We thank the Medical Research Council and the Arthritis and Rheumatism Council for supporting this research and Ciba/Geigy for supplying $ACTH^{1-24}$.

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*(Received October 6, 1976.*
*Revised November 11, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 433–434

# THE RELEASE OF PROLACTIN BY MEDROXYPROGESTERONE ACETATE IN HUMAN SUBJECTS

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Medroxyprogesterone acetate an injectable contraceptive when administered to four lactating women at a dose of 150 mg every three months significantly raised prolactin levels over those observed in four control women. Prolactin levels were higher at all times during 4–17 weeks in the drug-treated women, irrespective of whether the samples were collected before or after suckling. It was also observed that while suckling clearly released prolactin in both groups the difference was greater in the women receiving the injection, indicating that the drug enhanced the release of prolactin in response to the suckling stimulus.

**Introduction** A contraceptive agent which also caused an increase in the secretion of milk would be of great benefit, particularly in developing countries where mothers nurse their infants for long periods. It has been suggested that the injectable contraceptive, medroxyprogestone acetate (MPA) increases milk secretion in nursing mothers (Karim, Ammar, El Mahgoub, El Ganzoury, Fikri & Abdou, 1971; Koetswang, Chiemprasert & Kochananda, 1972), but there has been no detailed study of this attribute. The effect of MPA on the serum prolactin levels of lactating women has, therefore, been assessed in this investigation.

**Methods** The eight subjects aged 20–39 years were of similar socio-economic status and all had a previous history of successful lactation. The women nursed their infants at least five times a day for a minimum of 17 weeks. The 4 control women used non-hormonal means of contraception while the other 4 women were given an intramuscular injection of 150 mg MPA (Upjohn Ltd) at 2 and 14 weeks after parturition. The mothers were instructed not to nurse the babies for at least 3 h before coming to the clinic. At the clinic, a blood sample was taken and the infant was then immediately allowed to suck at one breast while milk was collected from the other by means of an Egnell breast pump. A second sample of blood was collected after 30 min of suckling. Similar pairs of blood samples were obtained at the end of weeks 1, 2, 4, 6, 8, 12, 15 and 17 of lactation.

The serum was immediately separated from the blood samples collected and stored at –40°C until assayed for prolactin by the radioimmunoassay procedure described by Hwang, Guyda & Friesen (1971). There is no cross-reactivity in this assay with thyrotrophin, growth hormone or human placental lactogen. The sensitivity was 1–5 ng/ml prolactin. The inter- and intra-assay variations were 15% and 10% respectively.

The prolactin levels at weeks 1 and 2 after birth were compared with the Wilcoxon Sum Test (Hollander & Wolfe, 1973). A regression analysis was used to determine the change in prolactin levels as lactation continued (Snedecor & Cochran, 1967). A paired $t$ test or a sign test were used to evaluate the differences in the prolactin levels (Snedecor & Cochran, 1967).

**Results** The results are shown in Table 1. The basal levels of circulating prolactin were those at weeks 1 and 2, before the MPA treatment had started. The apparent differences between the median prolactin levels of the control and MPA-treated women before and after suckling at weeks 1 and 2 were not significant ($P > 0.10$ for all four comparisons), indicating that the two groups of women were therefore comparable.

There were significant declines in prolactin levels as lactation proceeded except for the blood samples taken after suckling in the women treated with MPA. In the control women the decline was $8.56 \pm 0.67$ (s.e. mean) ng prolactin $\mathrm{ml}^{-1}$ $\mathrm{week}^{-1}$ before suckling and $16.01 \pm 6.40$ ng $\mathrm{ml}^{-1}$ $\mathrm{week}^{-1}$ after suckling.

Prolactin levels were higher at all times during weeks 4–17 in the MPA-treated women than in the controls, irrespective of whether the samples were collected before or after suckling.

**Discussion** The results obtained in this investigation suggest that the significantly raised levels of prolactin in the blood in the MPA-treated group at all times was a result of the MPA administration. Since it appears unlikely that the drug would interfere with the metabolism of normally released prolactin during lactation it appears that MPA acts either by directly releasing prolactin from the anterior pituitary or by inhibiting the normally occurring secretion of the prolactin inhibitory factor from the hypothalamus. Suckling clearly released prolactin in both groups, but

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**Table 1** Median (and range) prolactin levels (ng/ml serum) before and after suckling during lactation of women treated with medroxyprogesterone acetate (MPA)

| *Weeks after birth* | *Before suckling* | | *After suckling* | |
|---|---|---|---|---|
| | *Control** | *MPA** | *Control** | *MPA** |
| 1 | 112 (27–250) | 128 (28–285) | 125 (55–250) | 205 (160–285) |
| 2† | 80 (18–205) | 64 (29–170) | 188 (25–275) | 133 (85–165) |
| 4 | 54 (13–145) | 56 (30–90) | 130 (13–180) | 133 (60–195) |
| 6 | 44 (12–80) | 48 (33–95) | 72 (27–155) | 113 (47–165) |
| 8 | 26 (21–75) | 35 (11–75) | 46 (32–115) | 80 (31–100) |
| 12 | 20 (9–75) | 32 (11–40) | 37 (21–145) | 116 (21–255) |
| 15 | 19 (8–145) | 25 (15–45) | 28 (20–150) | 72 (45–140) |
| 17 | 20 (14–105) | 23 (16–50) | 44 (16–105) | 49 (40–140) |
| Value of regression | <0.05 | <0.02 | <0.05 | NS |
| Value of difference between groups | <0.02 | | <0.01 | |

* 4 women/group; † MPA treatment started after this sampling period.

the difference was greater in the women receiving MPA, suggesting that the drug not only induces release of prolactin but also enhances the release of prolactin in response to the suckling stimulus. These findings have two clinical implications. If the amenorrhoea and delayed return of ovulation associated with the use of MPA is the result of the release of prolactin then treatment with bromocryptine, a drug which suppresses the formation of prolactin may prevent the occurrence of these side effects. It is also possible that this pharmacological release of prolactin could be used to increase milk secretion where it is inadequate.

We thank Dr J.J. Schlesselman of the NICHHD, Bethesda U.S.A. and Mrs Yupha Onthuam of the WHO Research Team for the statistical analyses.

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*(Received November 27, 1976.)*

# PROCEEDINGS OF THE
# British Pharmacological Society

**5th–7th January, 1977**

NATIONAL INSTITUTE FOR MEDICAL RESEARCH,
MILL HILL

## COMMUNICATIONS

### Potentiation of angiotensin II-induced natriuresis by indomethacin in the rat

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High doses of angiotensin II (Ang-II) markedly decrease rat renal tubular electrolyte reabsorption thereby inducing natriuresis. (Malvin & Vander, 1967). In view of the recent demonstration by Malik & McGiff (1975) that Ang-II is a potent stimulant of prostaglandin formation in rat kidney we have investigated the involvement of prostaglandin biosynthesis in Ang-II-induced natriuresis in the rat, using indomethacin, a known inhibitor of prostaglandin synthesis in rat kidney (Armstrong, Blackwell, Flower, McGiff & Mullane, 1975).

In untreated rats Ang-II, (1 µg $kg^{-1}$ $min^{-1}$) increased urine flow from 19.8 ± 2.7 µl/min to 34.1 ± 3.4 µl/min ($n = 8$) within 20 minutes. Pretreatment of animals with indomethacin (10 mg/kg, orally), 3 h before anaesthesia potentiated the Ang-II response increasing urine flow almost 5-fold from 16.6 ± 3.3 to 81.5 ± 6.8 µl $min^{-1}$ $100^{-1}$ g. Similar changes were observed with sodium excretion which increased from 3.37 ± 0.37 to 5.4 ± 0.35 µEq $min^{-1}$ $100^{-1}$ g after angiotensin in untreated rats and from 2.42 ± 0.49 to 12.52 ± 1.58 µEq $min^{-1}$ $100^{-1}$ g in indomethacin pretreated animals.

Calcium excretion increased from 0.049 ± 0.005 to 0.101 ± 0.005 µEq $min^{-1}$ $100^{-1}$ g in the untreated group and from 0.040 ± 0.007 to 0.197 ± 0.005 µEq $min^{-1}$ $100^{-1}$ g following indomethacin pretreatment. Ang-II increased calcium excretion more markedly than sodium excretion resulting in a decreased Na/Ca-ratio. Pretreatment with indomethacin, however, resulted in an increase in the Na/Ca-ratio after Ang-II. Only small changes were observed in potassium excretion.

Malik and McGiff (1975) and Armstrong *et al.* (1975) demonstrated an increase in renal vascular resistance after intra-arterial injection of $PGE_2$ in the rat; consequently an attenuation of the Ang-II induced constriction of renal blood vessels might be expected after indomethacin pretreatment.

In our experiments, however, there was no significant difference in glomerular filtration rate or renal blood flow between untreated and indomethacin treated rats. Therefore, bearing in mind a possible redistribution of local intrarenal bloodflow, a decreased tubular reabsorption of sodium is likely to contribute to the potentiated natriuresis following Ang-II after indomethacin pretreatment, as suggested by the elevated Na/Ca-ratio.

The above results suggest that the natriuretic effect of Ang-II is enhanced following inhibition of intrarenal prostaglandin biosynthesis in the rat.

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## Production of 6-oxo-prostaglandin $F_{1\alpha}$ by rat, guinea-pig and sheep uteri, *in vitro*

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We have recently shown that 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) is the major prostaglandin produced following the incubation of pseudopregnant rat uterine homogenates (Fenwick, Jones, Naylor, Poyser & Wilson, 1977). We have now extended these studies to guinea-pig and sheep uteri. Whole uterine homogenates were incubated at 37°C for 90 min and the prostaglandins produced extracted by organic solvent (Poyser, 1972). Guinea-pig and rat extracts were further purified by silicic acid column chromatography. PGE and PGF fractions were obtained with 6-oxo-$PGF_{1\alpha}$ (and $PGD_2$) being present in the PGE fraction. Amounts of $PGE_2$ and $PGF_{2\alpha}$ produced were estimated by both radioimmunoassay (RIA) and gas chromatography-mass spectrometry (GC-MS).

Sheep uterine extracts were purified by straight-phase gel partition chromatography (Brash & Jones, 1974). The order of elution from the column is $PGD_2$ and 6-oxo-$PGF_{1\alpha}$, $PGE_2$, $PGF_{2\alpha}$. These substances were submitted to methyl ester (Me), n-butyloxime (BuO) and trimethylsilyl ether (TMS) derivitization for identification and estimation by GC-MS (see Table 1).

In the rat and sheep samples, 6-oxo-$PGF_{1\alpha}$ was the major product identified, whereas in the guinea-pig more $PGF_{2\alpha}$ than 6-oxo-$PGF_{1\alpha}$ was produced. Sheep uteri were able to use exogenous arachidonic acid as evidenced by the incorporation of radioactivity in the 6-oxo-$PGF_{1\alpha}$ molecule when [1–$^{14}$C] arachidonic acid was added to the homogenate. Also, deuterated 6-oxo-$PGF_{1\alpha}$ was formed when 5, 6, 8, 9, 11, 12, 14, 15-octadeutero-arachidonic acid was used as substrate. The deuterated 6-oxo-$PGF_{1\alpha}$ showed complete loss of one deuterium atom (from carbon 6) and partial loss of a second since the deuterium atom on carbon 5 is now adjacent to the 6-oxo group and is thus exchangeable with the medium. Recovered deuterated $PGF_{2\alpha}$ showed negligible loss of deuterium.

6-oxo-$PGF_{1\alpha}$ runs as a single substance on GC provided that the ketone group is protected by oxime of 6-oxo-$PGF_{1\alpha}$. This suggests that the cyclic ether can be produced from the hemiacetal form of 6-oxo-

**Table 1** Prostaglandin production by rat, guinea-pig and sheep uteri, *in vitro*

| *Species* | *Added Arachidonic Acid (μg/ml)* | *Amount of PGs recovered (ng/g)* 6-oxo-$PGF_{1\alpha}$ | $PGF_{2\alpha}$ | $PGE_2$ | $PGD_2$ |
|---|---|---|---|---|---|
| Rat (pooled pseudopregnant) | nil | 3000 | 800$^{+}$ | 200$^{+}$ | 30 |
| Guinea-pig (pooled, dioestrus) | nil | 250 | 600$^{+}$ | 100$^{+}$ | 20 |
| Sheep (pooled, nonpregnant) | 10 | 3200 | <50 | <100 | <100 |
| Sheep (pooled, nonpregnant) | 10 | 1800 | <10 | <20 | <20 |
| Sheep (pooled, nonpregnant) | 10 | 530 | 20 | 55 | <10 |

$^{+}$Values obtained by radioimmunoassay; other values from gas chromatography-mass spectrometry.

formation (though 2 oxime isomers are formed). If oximation is omitted and the Me-TMS derivative is formed, two peaks are seen on GC analysis. The major peak has a retention time (C value = 24.3) and mass spectrum identical to 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid (cyclic ether), as described by Pace-Asciak and Wolfe (1971) and derived from rat stomach homogenates. The second peak (C value = 25.7) is probably the open chain form $PGF_{1\alpha}$ during derivitization and gas chromatography and that it is not present in the original extracts. The biosynthetic route from arachidonic acid to 6-oxo-$F_{1\alpha}$ in the uterus is under investigation.

This study was supported by the Medical Research Council.

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# The potentiation of exogenous noradrenaline by prostaglandins $F_{2\alpha}$ $C_2$ and $D_2$ on the canine saphenous vein

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Kadowitz, Sweet & Brody (1971) have shown that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) potentiates the response of venous smooth muscle to exogenous noradrenaline (NA). $PGD_2$ acts similarly to $PGF_{2\alpha}$ on several preparations (Horton & Jones, 1974) whilst $PGC_2$ has similar actions to $PGE_2$ (Jones, Kane & Ungar, 1974). Experiments are reported here which compare the potencies of $PGF_{2\alpha}$, $PGD_2$ and $PGC_2$ on a single preparation.

The method used was a modification of that described by Webb-Peploe & Shepherd (1968) and was used to determine the venous response to exogenous NA before and during infusion of the various PGs. Both lateral saphenous veins were cannulated at the ankle and perfused separately, one serving as a control for the other. NA was injected proximal to the pump and once a dose dependent relationship was obtained, the PG infusion was commenced.

$PGF_{2\alpha}$ (1 µg/min) caused a significant potentiation of the response and the calculated potency ratio (R) from the pooled results of three preparations was 6. $PGD_2$ (1 µg/min) was not as potent (R = 3, from six preparations) and $PGC_2$ (1 µg/min) was the least potent (R = 2, from three preparations). Using linear regression analysis, the results during PG infusion (1 µg/min) were significantly different from control ($P < 0.05$). In the two preparations, $PGF_{2\alpha}$ (0.1 µg/min) was found to potentiate the response (R = 2). However, at 5 µg/min there were considerable baseline changes and no satisfactory tests were performed. At 5 µg/min $PGC_2$ caused similar baseline changes. Up to 10 µg/min, $PGD_2$ was apparently no more potent than at 1 µg/min and no baseline shift occurred.

The results with $PGF_{2\alpha}$ are largely in agreement with those of Kadowitz *et al.* (1971) and a similar potency ratio for $PGF_{2\alpha}$ (1 µg/min) has been obtained. We have found $PGD_2$ to be considerably less potent than $PGF_{2\alpha}$, whereas it is up to 60 times more potent than $PGF_{2\alpha}$ as a direct vasoconstrictor in the sheep (Jones, 1975). $PGC_2$ which from our results is only weakly active in potentiating the effects of NA, is in contrast a potent dilator of both resistance and capacitance vessels in the dog (Jones, Kane & Ungar, 1974).

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# Mutual enhancement of the effects of prostaglandin $E_1$ and theophylline on the Freund adjuvant-induced arthritis syndrome of rats

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Prostaglandin $E_1$ ($PGE_1$) counteracts the adjuvant-induced arthritis of rats (Zurier, Hoffstein & Weissmann, 1973). *In vitro,* $PGE_1$ suppresses lymphocyte function (Morley, 1974) and increases leucocytic cyclic AMP levels, the latter effect being enhanced by theophylline (Bourne, Lichtenstein & Melmon, 1972). The mutual effect of these two drugs has now been examined on the adjuvant arthritis of rats.

Male Lewis rats received 0.1 ml adjuvant (5 mg/ml *Mycobacterium butyricum* in liquid paraffin) in the left hind paw and acute inflammation was evaluated after 6 h by the increase in volume of the treated paw. Chronic inflammation (day 28) was measured by the increase in volume of the untreated paw. Malfunction in the latter condition was evaluated by a gait test and X-ray photographs of the tibiotarsal joint of the untreated paws were made on day 35. The latter two evaluations were carried out by observers who were unaware of the treatments. The rats were killed on day 35 and the adrenals and spleens removed and weighed. $PGE_1$ (0.75 mg/kg), theophylline (75 mg/kg) or both, were injected s.c. daily from the day of adjuvant administration until autopsy.

The results (Table 1) show that neither drug alone affected acute inflammation, but together they caused inhibition. Chronic inflammation was inhibited by each drug and combined treatment enhanced the effects. Theophylline failed to influence gait function or joint destruction, but $PGE_1$ maximally restored the former and partially counteracted the latter, which was further improved by theophylline. $PGE_1$ did not increase adrenals weight but markedly amplified the effect of theophylline. The effects of the drugs on spleen weight paralleled their inhibition of the chronic inflammation.

We propose that, *in vivo,* $PGE_1$ and theophylline each amplify the effect of the other by increasing cyclic AMP levels in splenic lymphocytes, resulting in enhanced suppression of adjuvant arthritis.

This work was sponsored by the Dutch Association to Combat Rheumatism.

**Table 1** Effects of $PGE_1$ and theophylline on several parameters of the adjuvant arthritis of the rat.

| | *Acute Inflammation† (treated paw % increase)††* | *Chronic Inflammation§ (untreated paw % increase)††* | *Spleen weight (mg)††* | *Adrenal weight (mg)††* | *Tibiotarsal joint destruction in untreated paw (%)* | *Malfunction of untreated paw (%)* |
|---|---|---|---|---|---|---|
| Adjuvant control | 109 ± 4 (12) | 63 ± 10 (12) | 247 ± 16 (5) | 20 ± 4 (5) | 83 (6) | 50 (6) |
| Theophylline 75 mg $kg^{-1}$ $day^{-1}$ s.c. | 110 ± 10 (12) | 42 ± 5* (11) | 189 ± 9** (7) | 31 ± 4* (7) | 67 (6) | 43 (7) |
| Prostaglandin $E_1$ 0.75 mg $kg^{-1}$ $day^{-1}$ s.c. | 117 ± 5 (12) | 40 ± 7* (12) | 188 ± 9** (6) | 21 ± 5 (6) | 17* (6) | 0**(6) |
| Theophylline plus Prostaglandin $E_1$ | 70 ± 7***(12) | 30 ± 7**(12) | 155 ± 9***(6) | 57 ± 8**(6) | 0***(5) | 15* (7) |

All values are means ± s.e.mean except the last two columns in which the values were obtained from quantal (all or none) tests. The number of observations are given in brackets. The acute and chronic inflammation values were derived from two separate experiments, other parameters were obtained from one experiment. † 6 h. †† corrected for 100 g body weight.

§ Day 28. Adjuvant controls received saline (1 ml $kg^{-1}$ $day^{-1}$) s.c. Significance versus control in the first 4 columns was calculated with the one tailed Student's *t*-test. In the last 2 columns significance versus control was derived from a $\chi^2$ test.

* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

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# Cyclic AMP production during adjuvant-induced arthritis in rats

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Following the observation that prostaglandin $E_1$ ($PGE_1$) and theophylline act synergistically in alleviating adjuvant arthritis in rats (Bonta, Parnham, van Vliet & Vincent, 1977), we have investigated cyclic AMP production during perfusion of arthritic paws.

Male Lewis rats received 0.1 ml complete or incomplete Freund's adjuvant in the left hind paw. After 10, 14, 18 or 22 days a stainless steel coaxial catheter was inserted, sub-cutaneously, during urethane anaesthesia, into the thigh of the right (chronic) hind leg until the tip covered the tibiotarsal joint. Left (acute, 6 h) metatarsal joints were perfused (s.c.) from 2 opposing needles. The catheter was tied in place and the joint perfused for 2 h with 6% dextran-saline at 0.2 ml/min, the perfusate being collected over ice. Two groups of arthritic rats were treated with either saline (1 ml $kg^{-1}$ $day^{-1}$ s.c.) or $PGE_1$ (0.5 mg $kg^{-1}$ $day^{-1}$ s.c.) on days 16 to 22 before perfusion on day 22. Total lipids were extracted from 4 ml of each perfusate with 10 ml chloroform: methanol (2 : 1) and the dextran emulsified with 2 × 10 ml ethanol. Following centrifugation at 2500 rev/min for 5 min, the supernatant was evaporated to dryness and resuspended in 350 µl water for duplicate assay of cyclic AMP by the method of Gilman (1970).

In the acute phase of adjuvant arthritis, despite a significant increase in paw volume, cyclic AMP levels were unaltered (Table 1). During the chronic phase

**Table 1** Cyclic AMP levels in perfusates of inflamed joints of rats with adjuvant-induced arthritis

| *Treatment* | *Acute inflammation†* | | *days after adjuvant injection* | *Chronic inflammation* | |
|---|---|---|---|---|---|
| | *cAMP (pmoles/h)††* | *% increase in paw volume††* | | *cAMP (pmoles/h)††* | *paw volume (ml)††* |
| Complete adjuvant (0.1 ml) | 59.4 ± 2.8 (3) | 94.3 ± 10.4*(4) | 10 | 83.1 (80.0–85.9) (2) | — |
| | | | 14 | 41.4 ± 8.9**(5) | — |
| | | | 18 | 30.1 ± 8.8**(4) | — |
| | | | 22 | 86.2 ± 31.0 (5) | — |
| Incomplete adjuvant (0.1 ml) | 53.0 ± 11.8 (4) | 63.4 ± 11.6 (4) | 22 | 111.9 ± 25.9 (3) | — |
| Complete adjuvant + saline (1 ml/kg s.c.)§ | — | — | 22 | 152.2 ± 32.0 (5) | 1.060 ± 0.069 (5) |
| Complete adjuvant + $PGE_1$ (0.5 mg/kg s.c.)§ | — | — | 22 | 212.2 ± 74.0 (5) | 1.198 ± 0.015***(5) |

All values are means ± s.e. mean except the cAMP value for day 10 where the range of values is given. The numbers of observations are given in brackets. † 6 h. †† corrected for 100 g body weight. § daily dose, days 16–22. Significance of differences in paw volume and of differences in cAMP values from day 22 incomplete adjuvant controls was calculated by Student's *t*-test.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

cyclic AMP levels decreased until day 18 but rose again between days 18 and 22, when paw volume was still increasing (Bonta *et al.*, this volume). $PGE_1$ ($0.5\ mg\ kg^{-1}\ day^{-1}$) did not significantly alter the late increase in cyclic AMP, though paw volume was increased.

These results suggest that, in adjuvant arthritis, gross changes in cyclic AMP levels at the inflammatory site cannot be correlated with the inflammatory response. They may reflect different changes in individual cell populations, as observed with lymphocyte subpopulations *in vitro* (Bach, 1975).

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## Actions of phospholipase-A on mast-cell histamine release and paw oedema in the rat

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Local administration of E-prostaglandins potentiates carrageenin-induced oedema formation in the rat paw (Moncada, Ferreira & Vane, 1973). A prostaglandin precursor, arachidonic acid, also potentiates carrageenin paw oedema and this action is abolished by administration of a non-steroid anti-inflammatory drug, indomethacin, known to inhibit prostaglandin synthesis (Lewis, Nelson & Sugrue, 1975). In the present work, we have investigated the effects on paw oedema of phospholipase-A (PL-A), an enzyme involved with the liberation of endogenous prostaglandin precursors, and have studied the actions of anti-inflammatory agents. However, since crude PL-A is known to release inflammatory mediators from rat mast cells (Thomas & Whittle, 1976) which therefore could lead to oedema formation, we have first compared the ability of PL-A obtained from various sources to liberate histamine from rat mast cells with their effects on rat paw oedema.

Mast cells were obtained by lavage of the rat peritoneal cavity with a modified buffer solution (pH7) and the histamine release following incubation (20 min at 37°C) was determined by fluorometric assay. Crude phospholipase-A from *vipera russellii* venom caused a dose-dependent release of histamine; a concentration of 20 µg/ml (0.1 unit/ml enzyme activity) gave a $75 \pm 7\%$ (mean ± s.e. mean, $n=5$) release of the total histamine content of the mast cells. However, the PL-As from other sources gave only a low histamine release; in five experiments, PL-A from *crotalus terrificus* venom (20 µg/ml; 6 units/ml) gave $9 \pm 2\%$ release, that from bee venom (20 µg/ml; 31 units/ml) gave $8 \pm 2\%$ release and the $PL\text{-}A_2$ from pig pancreas (20 µg/ml; 16 units/ml) $13 \pm 1\%$ release. Thus, the histamine-releasing activity was not correlated with PL-A enzyme activity, and may be due to presence of a lytic factor in the crude PL-A.

Changes in rat paw volume following subplantar injections were determined with a mercury-displacement plethysmograph (Van Arman, Begany, Miller & Pless, 1965). PL-A from *vipera russellii* (0.5–5 µg in 0.1 ml) gave a rapid marked rise in paw volume reaching a maximum after 30 min, as was found with the histamine liberator, compound 48/80 (1–10 µg in 0.1 ml). The PL-A from *crotalus* and bee venoms (5–20 µg) also caused an increase in paw volume following local injection, but were less effective. In contrast, pig pancreas $PL\text{-}A_2$ (5–20 µg) had no consistent effect on paw volume. However, this $PL\text{-}A_2$ (10 µg) significantly potentiated the increase in paw volume (by $212 \pm 28$ µl, $n=20$; after 1.5 h, $P<0.001$) following simultaneous subplantar administration of carrageenin (0.1 ml, 2% suspension). Pretreatment with indomethacin (15 mg/kg, s.c.; 1 h prior to carrageenin) in a dose causing 75% inhibition of the carrageenin-induced paw oedema, reduced this potentiation with $PL\text{-}A_2$ (to $92 \pm 10$ µl, $n=14$; $P<0.01$), whereas pretreatment with an equi-active anti-inflammatory dose of dexamethasone (100 µg/kg, s.c.) did not significantly alter this response ($177 \pm 33$ µl, $n=14$).

These results show that local administration of $PL\text{-}A_2$, like exogenous prostaglandins, can potentiate carrageenin-induced rat paw oedema. The ability of a prostaglandin synthetase inhibitor to reduce this response may suggest that endogenous prostaglandin formation is involved in the potentiation. The failure of dexamethasone to inhibit this $PL\text{-}A_2$ response could indicate that the anti-inflammatory steroids exert their effects at a stage prior to, or independent of, the involvement of $PL\text{-}A_2$ (see Gryglewski, 1976), although the present findings do not preclude the possibility of actions on the activation or release of endogenous $PL\text{-}A_2$.

This work was supported by the Medical Research Council.

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## Phospholipase $A_2$ activity of guinea-pig perfused lungs: stimulation and inhibition by anti-inflammatory steroids

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Guinea-pig isolated lungs release prostaglandin (PG) endoperoxides and thromboxane $A_2$ ($TXA_2$) into the perfusion fluid in response to stimuli including antigen challenge (Piper & Vane, 1969), mechanical trauma (Palmer, Piper & Vane, 1973), bradykinin or arachidonic acid (Vargaftig & Dao Hai, 1972) and rabbit aorta contracting substance – releasing factor (RCS-RF; Piper & Vane, 1969; Nijkamp, Flower, Moncada & Vane, 1976). Nijkamp *et al.* (1976) demonstrated that in relation to their anti-inflammatory potency, corticosteroids inhibited the generation of $TXA_2$ induced by RCS-RF, but not that due to the precursor arachidonic acid. Thus, inhibition of the release of arachidonic acid could be related to the therapeutic action of these steroids. Flower & Blackwell (1976) demonstrated that arachidonic acid was released from cellular phosphatides in response to similar stimuli, and this led us to speculate that agents which release $TXA_2$ from lungs do so by "activating" phospholipase $A_2$.

For these experiments, the guinea-pig perfused lungs and cascade superfusion apparatus were prepared and $TXA_2$ generation was measured as previously described (Nijkamp *et al.*, 1976). For assay of phospholipase $A_2$ activity, a mixture of 18.0 nmoles 2-acyl ([$^3$H]-oleoyl) phosphatidylcholine and 1.8 nmoles [$^{14}$C] oleic acid was injected into the pulmonary artery. The perfusate was collected for 7 min and the labelled fatty acids selectively extracted at pH 8.0 with 10 ml *n*-hexane. The solvent was evaporated to dryness and the $^3$H/$^{14}$C ratio estimated by conventional liquid scintillation counting techniques.

When injected into the pulmonary artery, histamine (2–5 µg), RCS-RF (5–10 u), bradykinin (1–5 µg) and arachidonic acid (1–5 µg) caused a release of PG endoperoxides and $TXA_2$. Release of $TXA_2$ was blocked by indomethacin (1 µg/ml). The release induced by histamine or RCS-RF was also blocked by dexamethasone ($ID_{50}$ 1.5 µg/ml) and hydrocortisone ($ID_{50}$ 33 µg/ml).

There was a small (1–3%) basal hydrolysis of the labelled phosphatide by the perfused lung, which increased gradually with time. This hydrolysis was inhibited by mepacrine (20 µg/ml), procaine (40 µg/ml), betamethasone (2 µg/ml), dexamethasone (2 µg/ml) and hydrocortisone (50 µg/ml). The steroids exhibited a time-dependent inhibition, the maximum effect occurring after 30 min infusion. Histamine (2 µg), RCS-RF (5 u) and bradykinin (1 µg) stimulated phospholipid hydrolysis by 150–300%. Steroids and mepacrine blocked (60–90%) the stimulation due to histamine and RCS-RF but had only a small effect (10–20%) on the bradykinin stimulation.

In homogenates of guinea-pig lung, phospholipase $A_2$ activity was inhibited by mepacrine and procaine. Steroids were without effect, indicating that these agents require intact cells to function effectively.

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# Microvascular responses produced by the prostaglandin endoperoxide $PGG_2$ *in vivo*

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We have investigated the effects of $PGG_2$ on microvessels *in vivo* using microscopic and isotopic techniques.

The hamster cheek pouch preparation was set up as described by Lewis & Westwick (1975). Changes in arteriolar diameter (25–45 μm) were monitored continuously using a photometric technique (Hutchings, Lewis, Sabikowski & Westwick, 1976).

$PGG_2$ (stored at –20°C in acetone, 500 μg/ml) was diluted either in saline or acetone at 0°C and applied immediately (0.5–5 μl) via a microsyringe onto the vessel being viewed under the microscope. The $PGG_2$ solutions were assayed simultaneously using isolated vascular strips (Bunting, Moncada & Vane, 1976).

On arterioles having a low vascular tone, $PGG_2$ (25–100 ng) produced a short lasting vasoconstriction reaching a maximum at 30 to 60 s, e.g. $PGG_2$ (25 ng) produced $93 \pm 2\%$ constriction (mean ± s.e. mean, $n=6$). Repeated doses of $PGG_2$ rapidly induced tachyphylaxis which was not observed on the isolated vascular strips. In comparison, noradrenaline (NA) produced $57 \pm 3\%$ ($n=5$), and $80 \pm 2\%$ ($n=5$) vasoconstriction in doses of 0.1 ng and 1.0 ng respectively. Doses of NA equiactive with $PGG_2$ were of longer duration and were not tachyphylactic.

On vessels having a high tone induced by a continuous superfusion of NA (0.5 ng $ml^{-1}$ $min^{-1}$), $PGG_2$ produced a much smaller vasoconstriction ($PGG_2$, 100 ng, $45 \pm 7\%$, $n=4$) followed by a protracted phase of strong vasodilatation (60–100% with 25–100 ng $PGG_2$). Control applications of cold saline or acetone produced a small vasoconstriction ($4 \pm 2\%$, $n=6$). In these concentrations of $PGG_2$ white body formation was not observed.

Similar two-phase vascular responses to $PGG_2$ were observed in rabbit skin using a $^{133}Xe$ clearance technique to measure blood flow (Lewis, Peck, Williams & Young, 1975). $PGG_2$ in acetone mixed with $^{133}Xe$ in saline (250 ng $PGG_2$/50 μl injection) was injected into dorsal skin and $^{133}Xe$ washout monitored every 10 s for 15 min using a $\gamma$-detector. The means of six sets of observations were compared with six controls (acetone/$^{133}Xe$ in saline). Reduced flow (i.e. reduced $^{133}Xe$ washout) was apparent for the first 70 seconds. The count at 70 s as a percentage of the initial count was: $PGG_2$ $89.15 \pm 0.85\%$; control, $82.10 \pm 1.29\%$. This was followed by a protracted phase of increased flow; the count at 360 s was: $PGG_2$, $8.61 \pm 1.22\%$; control, $20.88 \pm 0.98\%$). The initial reduced flow was less in magnitude and duration than that produced by 10 ng of noradrenaline.

Because of the dominance of the vasodilatation produced by $PGG_2$, a pro-inflammatory effect on bradykinin-induced plasma exudation would be expected (Williams, 1976a). Using the method of Williams (1976b) marked potentiation was observed with $PGG_2$. The mean plasma accumulation per injection site produced by bradykinin (500 ng dose) was $15.99 \pm 2.71$ μl, and by $PGG_2$ (250 ng) $5.60 \pm 2.13$ μl, whereas for the mixture of bradykinin and $PGG_2$ the accumulation was $57.02 \pm 5.35$ μl ($n=6$).

The significance of these observations depends on the site and nature of $PGG_2$ production under physiological or pathological conditions. Under the conditions of our experiments, $PGG_2$ produced a transient vasoconstriction followed by a protracted phase of vasodilatation. The dilator effect results in potentiation of bradykinin-induced exudation. The observed effects could be due to $PGG_2$ itself or products of $PGG_2$.

This work was supported by the Medical Research Council and Ciba-Geigy Ltd. We thank Professor B. Samuelsson for supplying the $PGG_2$.

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# Prostaglandin X, the vascular metabolite of arachidonic acid responsible for relaxation of bovine coronary artery strips

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Both arachidonic acid and prostaglandin $E_2$ ($PGE_2$) dilate the coronary vascular bed of most species (see Nakano, 1973), and thus it has been assumed that $PGE_2$ is the metabolite of arachidonic acid which is responsible for coronary vasodilatation. However, the coronary vascular bed of the rabbit does not respond to $PGE_2$ (Block, Feinberg, Herbaczynska-Cedro & Vane, 1975) and spirally cut strips of bovine and human coronary arteries are contracted by $PGE_2$ (and $PGF_{2\alpha}$), whereas arachidonic acid causes relaxation of the same preparations (Kulkarni, Roberts & Needleman, 1976). Recently, we have discovered an unstable intermediate in the metabolism of arachidonic acid (Moncada, Gryglewski, Bunting & Vane, 1976), which we have called PGX. This intermediate is synthesized by an enzyme present in the microsomal fractions of blood vessels and has a striking combination of biological activities including relaxation of vascular smooth muscle and potent inhibition of platelet aggregation. We have now investigated the involvement of PGX in the arachidonic acid-induced relaxation of coronary artery strips of the ox.

The left ramus descendens of the coronary artery and some of its major branches were excised from ox hearts within 2–3 h of slaughter, and generally stored in Krebs' solution at 4°C for 1–2 days before use. Helically•cut strips (1–2 cm long) were suspended (initial tension of 2 g) in oxygenated Krebs' bicarbonate solution at 37°C, changes in tension were recorded isometrically on a Grass polygraph.

Arachidonic acid relaxed coronary artery strips (threshold concentration 10–100 μg/ml) but they were contracted by $PGE_2$ (50–200 ng/ml). The effects of exogenous prostaglandin endoperoxide ($PGH_2$) were variable. Most strips were relaxed by $PGH_2$ (20–200 ng/ml). In a few, the relaxation was preceded by a transient contraction, and in others there was only a contraction. Thromboxane $A_2$ (generated by incubation of $PGH_2$ with horse platelet microsomes for 2 min at 0°C) caused strong contraction in all strips, the potency being more than double that of $PGE_2$. Exogenous PGX (20–500 ng/ml) always caused a dose-dependent relaxation, generally being equipotent with $PGH_2$.

Treatment of arterial strips with indomethacin (1–2 μg/ml) abolished or substantially reduced the relaxation induced by arachidonic acid, but did not affect the small relaxations sometimes produced by high concentrations (50–200 μg/ml) of linoleic acid. Relaxations induced by exogenous PGX were increased after indomethacin, whereas the non-specific relaxation produced by erthrityl trinitrate (1 μg/ml) was not altered.

These findings strongly suggest that PGX is responsible for the relaxation induced by arachidonic acid in bovine coronary artery strips. As prostaglandin endoperoxides are avidly converted into PGX by vascular tissue, and this represents the major pathway of the vascular metabolism of the endoperoxides in man and other species (unpublished observations), it is likely that this intermediate is the active vasodilator principle in the coronary vascular bed of most species.

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# Release of prostaglandins and rabbit aorta contracting substance (RCS) from guinea-pig lung by slow reacting substance of anaphylaxis (SRS-A)

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Guinea-pig lungs previously sensitized to ovalbumin respond to challenge by the release of several mediators including histamine, SRS-A, RCS (thromboxane $A_2$ ($TXA_2$) and prostaglandins $G_2$ and $H_2$), releasing factor for RCS (RCS-RF) and other prostaglandin-like substances (Piper & Vane, 1969).

SRS-A was prepared from indomethacin-treated isolated perfused lungs from sensitized guinea-pigs as previously described (Engineer, Piper & Sirois, 1976). Histamine was removed and SRS-A concentrated by adsorption onto activated charcoal and elution with 80% ethanol. After evaporation under vacuum the SRS-A was freeze-dried.

Lungs from either sensitized or unsensitized guinea-pigs were perfused with Tyrodes solution via the pulmonary artery at 5 ml/min and the effluent superfused over strips of smooth muscle from guinea-pig ileum, rabbit aorta, rabbit coeliac artery, rat stomach strip and chick rectum. The assay tissues were blocked with mepyramine and hyoscine.

When crude SRS-A was injected directly over the assay tissues it caused contraction of guinea-pig ileum and rat stomach strip but when injected into the pulmonary artery all the assay tissues contracted showing the passage of SRS-A through the pulmonary circulation and the release of RCS and prostaglandin-like substances from the lungs. Following injection of SRS-A into the lungs, RCS-induced contraction of rabbit aorta was matched by injecting $PGG_2$ over the tissues. Repeated injections of SRS-A released similar amounts of RCS from lungs. Any RCS-RF which might have been present in the samples of SRS-A was destroyed by boiling for 15 min (Flower, Harvey, Moncada, Nijkamp & Vane, 1976) followed by centrifugation for 5 minutes. The biological activity of SRS-A and its ability to release RCS and prostaglandins was unchanged by boiling. Incubation of SRS-A with arylsulphatase for 1 h destroyed the biological activity (Orange, Murphy & Austen, 1974); if the enzyme activity was terminated by boiling, the release of RCS was prevented, but in the absence of boiling there was sometimes a small release, probably due to contamination by RCS-RF. The release of RCS and prostaglandin-like substances by SRS-A was prevented by treating the lungs with indomethacin (1–5 μg/ml) or with dexamethasone (4–40 μg/ml). Furthermore, treatment of the lungs for 15 min with the SRS-A antagonist FPL-55712 (for ref. see Augstein, Lee, Sheard & Tattersall, 1973) (0.1–1.0 μg/ml) also prevented the release of RCS by SRS-A.

These results (a) strengthen the hypothesis that SRS-A released in anaphylaxis (Engineer *et al.*, 1976) might in turn release prostaglandin-related materials to inhibit further release of mediators, and (b) could explain the mechanism of action of steroid and non-steroid anti-inflammatory drugs in acute immunological reactions.

We wish to thank the Medical Research Council, Fisons Ltd., and the Conseil de la Recherche en Santé du Québec for grants.

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# The relationship between prostaglandin-like substances and SRS-A released from immunologically challenged lungs

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Although the biological activities of certain prostaglandins (PGs), particularly of the F series and slow reacting substance in anaphylaxis (SRS-A) on bronchial smooth muscle are similar, they appear to derive from two different biological precursors. PGs, thromboxanes and hydroxy fatty acids are formed from arachidonic acid by two enzymes, a cyclo-oxygenase and a lipoxygenase, both of which are inhibited by the competitive substrate, eicosatetraynoic acid (TYA). This compound reduced the PGs released on immunological challenge of guinea-pig lungs but did not modify SRS-A release, in contrast to disodium cromoglycate which had the reverse effect (Dawson & Tomlinson, 1974).

Walker (1973) has shown that some non-steriodal anti-inflammatory compounds increase SRS-A release, although these compounds are known to be inhibitors of PG synthetase (cyclo-oxygenase) under many conditions (Vane, 1971). The relationship between PG and SRS-A release has been further analysed using these compounds in preparations derived from passively sensitized human lung and actively sensitized guinea-pig lung (Sheard, Killingback & Blair, 1968; Brocklehurst, 1960).

Indomethacin (1 µg/ml), TYA (10 µg/ml) and aspirin (25 µg/ml) inhibited a microsomal preparation of PG synthetase from guinea-pig lung by 50%, whilst phenylbutazone (10 µg/ml) was virtually ineffective. It was not possible to produce a consistent preparation of this enzyme from human lung. At the same concentrations, the three anti-inflammatory compounds potentiated the release of SRS-A by at least 50% from both human and guinea-pig lungs, but had little effect on histamine release. TYA had no effect on SRS-A or histamine release.

$PGF_{2a}$ and thromboxane $B_2$ ($TXB_2$) increased the release of SRS-A from challenged guinea-pig lung whilst $PGE_2$ reduced it (Table 1). The levels of the parent PGs released on challenge are low, but that of $TXB_2$ reaches 1 µg/ml (Dawson, Boot, Cockerill, Mallen & Osborne, 1976). It appears that the most important member of the PG family in the modulation of SRS-A release is $TXB_2$. The data further lead to the speculation that compounds such as indomethacin may preferentially inhibit the formation of parent PGs, leaving the thromboxane pathway from arachidonic acid relatively untouched. $PGE_2$ and $PGF_{2a}$ are known to modify the adenyl and guanyl cyclase systems in lung tissue, which have been shown to regulate mediator release. Experiments to examine the effects of $TXB_2$ on the cyclic nucleotides and to analyse PG and Tx release in the presence of non-steroidal anti-inflammatory drugs are in progress.

**Table 1** Effect of prostaglandin-like substances on SRS-A release from immunologically challenged guinea-pig lung. ($n = 9$)

| *concentration* | *% change in SRS-A release (mean ± s.e. mean) in the presence of* | | |
|---|---|---|---|
| *(µg/ml)* | *$PGE_2$* | *$PGF_{2a}$* | *$TxB_2$* |
| 0.1 | 0 | | 0 |
| 1.0 | −20 ± 15 | +21 ± 12 | +49 ± 22 |
| 10.0 | −29 ± 6 | +40 ± 10 | +63 ± 34 |

+ = increased release; − = reduced release

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# Hydrostatic pulmonary oedema is not a stimulus for prostaglandin synthesis in isolated, perfused lungs

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Isolated lungs can be induced to synthetize and release prostaglandins (PGs) by a variety of mechanical and chemical stimuli (Piper & Vane, 1971). It has been suggested that the stimulus for PG synthesis may be distortion of cell membranes (Piper & Vane, 1971). Said & Yoshida (1974) have reported release of PGs during formation of oedema in isolated cat lungs. In their experiments oedema was induced by prolongation of perfusion time. This is, however, a non-specific method of induction. Thus PG synthesis might occur secondary to cellular injury, which also might be responsible for the pulmonary oedema.

In the present experiments we caused lung oedema by elevating the hydrostatic pressure in the pulmonary circulation in order to study the effect of vascular distention and increased extravascular lung water on pulmonary PG-synthesis.

Isolated lungs were prepared as described by Hauge, Lunde & Waaler (1966). The following combinations of species and perfusates were used: 5 rabbit lungs – horse plasma; 2 cat lungs – horse plasma; 2 cat lungs – cat plasma; 2 cat lungs – cat whole blood; 1 guinea-pig lung – Krebs Ringer solution and 2 guinea-pig lungs – horse plasma. The lungs were perfused (37°C) in a recirculating system with constant volume inflow (approximately 150 ml/min for cat and rabbit lungs, 20–30 ml/min for guinea-pig lungs). They were ventilated with 5% $CO_2$ in air at a constant tidal volume. Ten ml/min of the venous effluent was pumped to superfuse a rat stomach strip, a rat colon and a chick rectum for the continuous bioassay of PGs (Vane, 1969). The superfusate was then returned to the lung perfusion circuit. The tissues were sensitive to calibrating doses of 1 ng/ml $PGE_2$ and $PGF_{2\alpha}$.

Pulmonary oedema was induced by elevating the outflow pressure of the lungs to 10–30 mmHg for 10–70 minutes. In all lungs this manoeuvre caused gross alveolar oedema as evidenced by cessation of ventilatory movement, translucent appearance of lungs and foam in the trachea.

In none of the 14 experiments did raised outflow pressure or oedema development cause any release of PGs which could be detected on the tissues. Theoretically, PG-release of less than 0.5–1 ng/ml might have escaped detection. However, in four experiments, serial radioimmunological determinations of $PGF_{2\alpha}$ were performed on extracts of the perfusate. These experiments verified the findings with bioassay that no PGs were released during raised outflow pressure or subsequent oedema.

In conclusion, the present experiments suggest that neither vascular distension nor oedema is a stimulus for increased synthesis of prostaglandins in isolated, perfused lungs.

We are grateful to Dr Kaare M. Gautvik for the radioimmunoassay of $PGF_{2\alpha}$.

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## Effects of synthetic prostaglandin analogues on platelet aggregation and secretion

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Stable synthetic analogues of prostaglandin $E_2$ ($PGE_2$) and prostaglandin $H_2$ ($PGH_2$) induce platelet aggregation (Corey, Nicolau, Machida, Malmsten & Samuelsson, 1975; Gordon & MacIntyre, 1976), but the platelet responses to these agents have not been fully characterized. We have now compared the effects of 11-deoxy-15(RS)-15-methyl $PGE_2$ (Wy 17,186), 16,16 dimethyl $PGE_2$, and a 9,11-azo-prostanoid analogue of $PGH_2$ (azo-$PGH_2$) on platelet aggregation and secretion, and have investigated the effects of selected inhibitors on these responses.

Platelet aggregation was measured photometrically in 0.1 ml samples of human citrated platelet-rich plasma (PRP) pre-labelled with [$^{14}$C]-5-hydroxytryptamine (5-HT) and [$^3$H]-adenine. The reaction was terminated by the addition of 4 volumes ice-cold 0.4% w/v EDTA in iso-osmotic saline, followed by rapid centrifugation (15,000 $g$:30 s), and sub-samples of the platelet-free supernatant were taken for liquid scintillation counting and for fluorimetric measurement of $\beta$-N-acetyl glucosaminidase release. For inhibitor studies, drugs or an equal volume of solvent were pre-incubated in PRP at 37°C before measurement of aggregation and release.

Each compound induced a dose-dependent aggregation response, which was reversible ('primary aggregation') at low concentrations (Azo-$PGH_2$ $\leqslant$ 0.05 $\mu$M; 16,16 dimethyl $PGE_2$ $\leqslant$ 3 $\mu$M; Wy 17,186 $\leqslant$ 6 $\mu$M) and irreversible ('secondary aggregation') at higher concentrations. Intermediate concentrations produced biphasic responses. No release of $\beta$-N-acetyl glucosaminidase was observed even with high concentrations of agonist, but irreversible aggregation responses were accompanied by dose-dependent release (up to 50%) of [$^{14}$C]-5-HT, with less than 10% release of [$^3$H]-adenine. No [$^{14}$C]-5-HT was released during primary aggregation. Aggregation and 5-HT release were inhibited by agents ($PGE_1$ 0.1 µM; $PGD_2$ 0.1 µM; adenosine 1 µM) which increase intracellular cyclic AMP, and by dibutyryl cyclic AMP (0.5 mM). Secondary aggregation and the associated release of 5-HT were blocked by indomethacin (0.1 mM), but this inhibitory effect could be readily overcome by increasing the concentration of agonist. 2-n-amylthio-AMP (10–100 µM) blocked secondary but not primary aggregation responses. 2-n-amylthio-AMP is apparently a specific ADP antagonist: it inhibits primary aggregation induced by ADP but not by adrenaline, vasopressin or 5-HT, and its inhibitory effect (unlike that of $PGD_2$, $PGE_1$ or adenosine) is not blocked by 9-(tetrahydro-2 furyl) adenine (SQ 22536), an inhibitor of platelet adenylate cyclase (Harris, Phillips & Goldenberg, 1975).

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The actions of the prostaglandin analogues Wy 17,186, 16,16 dimethyl $PGE_2$ and azo-$PGH_2$ are similar, and are more complex than previously recognized. They directly induce a primary aggregation response and stimulate secondary aggregation and release by an indomethacin sensitive process, possibly triggered by platelet-platelet contact in the primary phase. In addition, they induce secondary aggregation and release by an indomethacin insensitive process. The secondary aggregation responses appear to be mediated by released ADP. In these respects, the complicated behaviour of these compounds resembles that of the native endoperoxides (Salzman, unpublished observations).

We thank Dr D. Harris, Squibb, for SQ 22,536; Dr K. Kikugawa, Kohjin Company for 2-n-amylthio AMP; Dr R.L. Fenichel, Wyeth, for Wy 17,186; and Dr J.E. Pike, Upjohn Company, for the other prostaglandins. This study was supported by grants from the M.R.C., the Arthritis and Rheumatism Council and the American N.H.L.I.

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## Levels of prostaglandin F and E in cerebrospinal fluid of cats during pyrogen-induced fever

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Prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) occur in cat brain (Horton & Main, 1967) however, Feldberg, Gupta, Milton & Wendlandt (1973) using bioassay failed to detect either prostaglandin in cat cerebral spinal fluid (CSF). Since $PGF_{2\alpha}$ is pyrogenic in cats (Ewen, Milton & Smith, 1976) we have measured the levels of PGF in the CSF of afebrile and febrile animals by the more sensitive techniques of radioimmunoassay.

Pyrogen (the O-somatic antigen of *Shigella dysenteriae*) dissolved in sterile, pyrogen-free 0.9% saline was injected intravenously (20 µg/kg) in a volume not exceeding 0.4 ml. Control animals were injected with a similar volume of sterile 0.9% saline. Two samples of CSF (approximately 0.5 ml) were collected from the cisterna magna before injection and four samples were collected afterwards. The interval between successive CSF samples was 75 minutes. Rectal temperature was recorded continuously.

Samples of CSF were extracted with ethyl acetate and assayed for PGE (after alkaline conversion to prostaglandin B) and PGF by a double antibody technique (Dighe, Emslie, Henderson, Simon & Rutherford, 1975) without prior separation.

Two distinct types of febrile responses to pyrogen injection were observed. In some animals which had not previously received pyrogen the fever developed after a long latency (90 min), whereas in animals which had received pyrogen before, the fever developed after a short latency (30 min). The level of PGF and PGE measured in CSF taken during the two types of fever and during control experiments are shown in the Table.

In the short latency fevers significant increases of both PGEs and PGFs which paralleled the increases in deep body temperature were seen, and these results are compatible with both prostaglandins having a role

**Table 1** Control and febrile PGE and PGF levels (in pg/ml) in cat CSF

| | *Sample No.* | *Control* | *Long latency* | *Short latency* |
|---|---|---|---|---|
| PGE | 1 | 137±17 | 144± 24 | 180± 48 |
| | 2 | 137±17 | 124± 4 | 185± 58 |
| | 3 | 138±18 | 126± 6 | 1756±1355** |
| | 4 | 142±11 | 259±101 | 455± 193** |
| | 5 | 129±10 | 331±154 | 1773± 682** |
| PGF | 1 | 84± 4 | 80 | 109± 39 |
| | 2 | 92± 8 | 93± 13 | 130± 21 |
| | 3 | 115±24 | 210± 56 | 759± 174*** |
| | 4 | 116±35 | 181± 56 | 1303± 400*** |
| | 5 | 80 | 142± 42 | 1428± 578*** |
| | 6 | 115±35 | 80 | 769± 170*** |

Comparison with control values.
Students 't' test *** $P<0.025$
** $P<0.05$
Values are the mean ± s.e. mean of 4 observations. Controls $n = 8$.

in pyrogen-induced fever. However, it should be realized that the precursors of these prostaglandins, namely prostaglandins G and H and the thromboxanes are themselves pharmacologically-active and may also be involved in fever.

During the long latency fevers, no significant increases in either the PGE or the PGF levels were found. The reasons for these differences between the short latency and long latency responses have not yet been determined.

This work was supported by a grant from the Medical Research Council. The O-somatic antigen of *Shigella dysenteriae* was kindly supplied by W.H.O. Laboratories, Hampstead, and prostaglandins by Dr John Pike, Upjohn Company.

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## Sex differences in guinea-pig brain prostaglandins and the effect of indomethacin

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Adult albino guinea-pigs were stunned and their brains removed quickly. Groups of 2–6 brains were pooled, cut into approximately 2 mm cubes and washed thoroughly with Krebs solution. Samples (1–5 g) were homogenized (30 s; immediately or after incubation at 37°C) in Krebs solution alone or with indomethacin or acid ethanol (Bennett, Stamford & Unger, 1973). The prostaglandin (PG)-like material was extracted (Unger, Stamford & Bennett, 1971) and assayed on rat gastric fundus (Bennett *et al.*, 1973). Results were compared using the Mann-Whitney U test unless otherwise stated.

Using Krebs solution alone the biological activities (medians and semiquartile ranges, ng $PGE_2$ equivalents/g fresh tissue) were: male 5.8 (4.5 to 17)

$n=8$; female 14 (11.5–22) $n=8$ ($2P=0.19$) after immediate homogenization, and male 7.9 (6.6 to 11) $n=8$; female 10.6 (9.3 to 22) $n=7$ after 30 min incubation. There was less activity in acid ethanol than in Krebs solution homogenates (−71% (−55 to −86), 5 male, 4 female, $2P<0.01$, Wilcoxon test).

With female brain the PG-like activity extracted after immediate homogenization with indomethacin (1 or 10 $\mu$g/ml) was always less than controls, (−67% (−63 to −90) $n=5$, and −33 to −65% $n=4$ respectively). In male brains, however, the effect of indomethacin was concentration-dependent: 1 $\mu$g/ml increased the activity in 5/6 experiments by 83% (38 to 120) (cf. female, $2P=0.03$), but 10 $\mu$g/ml caused inhibition (−28 to −91%, $n=4$). The difference did not seem primarily due to drug penetration or substrate availability. After 30 min incubation with 1 $\mu$g/ml indomethacin, biological activity was less in all female brains (−36% (−29 to −52) $n=7$) but the effect in male brains varied (−7% (20 to −13) $n=7$) (cf. female $2P=0.02$). With 40 or 400 $\mu$g/ml arachidonic acid in the incubate, 1 $\mu$g/ml indomethacin decreased biological activity in 3 female brains (−14 to −66%) but increased activity in 3/4 male brains (0 to 250%).

Extracts of brain homogenized in Krebs solution were chromatographed for group separation of PG (Stamford & Unger, 1972). In female brains only material running as PGE was detected ($n=4$) whereas in male brains PGF-like or PGE+PGF-like material was found ($n=5$) (cf. female $2P=0.016$, Fisher's exact probability test).

Brocklehurst & Dawson (1974) obtained stimulation of PG synthesis with low concentrations of indomethacin, and biological activity sometimes increased in gastrointestinal tissues homogenized with low concentrations of non-steroidal anti-inflammatory drugs (Bennett, Fox & Stamford, 1973). Apart from sex hormones, male and female guinea-pig brains might differ in co-factors for PG synthesis; arachidonic acid or noradrenaline tended to increase thromboxane $B_2$ but prevented $PGE_2$ and $F_{2\alpha}$ formation (Wolfe, Rostworowski & Marion, 1976). Alternatively, in male brain, indomethacin might increase the estimated activity by enhancing generation of PGE (to which the bioassay is more sensitive) at the expense of PGF or thromboxanes. Wolfe, Pappius & Marion (1976) found that $PGF_{2\alpha}$ production was more inhibited by indomethacin than $PGE_2$ production in rat brain.

We thank the Migraine Trust and Wellcome Trust for support.

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# Effect of colchicine and lumicolchicine on learning in goldfish

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Cronly-Dillon, Carden and Birks (1974) have reported that intracranial (i.c.) injections of colchicine interfere with long-term memory in goldfish (*Carassius auratus*). Fish were trained in a shuttlebox using an active avoidance task (Agranoff, 1967) in which they were taught to associate a light (CS) with an electric shock (UCS). Using a comparable technique, we have studied the effects of colchicine on goldfish over a longer time period and have, in addition, compared the effects of colchicine with those of its isomer, lumicolchicine.

Fish were trained in a shuttlebox (Aim-Biosciences Ltd.) and the performance of fish receiving 10 $\mu$g colchicine or lumicolchicine compared with controls receiving vehicle (Youngs Teleost Ringer solution).

The fish (6–8 g) were injected (10 µl i.c.) under anaesthesia tricaine–methane–sulphonate (MS222, Sandoz) and left to recover for 6 h before the training session. Training was carried out in the dark for 5 days (20 trials/day) at a constant temperature of 18°C. Performance was judged by the number of responses of each fish to the CS during the training period. Results were analysed by ranking tests and values of $P < 0.05$ were taken as significant. After 5 days, the performance of the colchicine injected fish was significantly poorer than that of controls, whereas that of fish injected with lumicolchicine was not significantly different from controls. All fish significantly improved their performance during the 5 days of training.

Other fish pretreated in a similar manner were used to assess unconditioned behaviour. The incidence of spontaneous crossings between chambers of the shuttlebox did not significantly differ between any two groups.

To try to trace the distribution of colchicine, groups of 4 fish were injected with [ring C-methoxyl-$^3$H] Colchicine (Radiochemical Centre) and killed at intervals over 5 days. No attempt was made to follow the metabolic fate of the injected tritium, but radioactivity was detected throughout the brains of all fish. At 5 days, the mean d.p.m./g of whole brain was similar to that at 24 h, but in liver, radioactivity was not detected beyond 24 hours.

Colchicine is an alkaloid of well documented action. It is known to disrupt neurotubule structure, probably by combining with soluble protein subunits, whereas lumicolchicine does not bind to neurotubules (Banks & Till, 1975). It is speculated that the observed behavioural effects of colchicine may be due to the disruption of the neurotubule system. That the observed effects may be due to general toxic actions seems unlikely since unconditioned colchicine-injected fish performed as well as controls.

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## The 'wet dog shake' behaviour in the rat and 5-hydroxytryptamine

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The 'Wet Dog' shake (WDS) in the rat is a paroxistic shudder of the head, neck and trunk, reminiscent of the purposeful movement seen in dogs. This behaviour has been reported after various physiological stimuli, following morphine withdrawal or injection of thyrotrophin-releasing hormone (Wei, Sigel, Loh & Way, 1975). The neuropharmacology of the WDS is not clear, although with all the stimuli there is the suggestion of the possible involvement of central 5-hydroxytryptaminergic mechanisms.

We have investigated the relationship between WDS response in the rat and central 5-hydroxytryptamine (5-HT) function. Administration of 5-hydroxytryptophan (5-HTP) (25–150 mg/kg, s.c.) in combination with a peripheral decarboxylase inhibitor (carbidopa, 25 mg/kg, i.p. 30 min beforehand) induced this behaviour in a dose-dependent manner. The WDS response began 15 min after 5-HTP injection, reaches a peak at 2 h and receded until the 6th hour. High doses of 5-HTP (200 mg/kg) were accompanied by stereotyped sniffing, padding of the forepaws and head weaving.

5-HTP-induced WDS was blocked by the 5-HT antagonists methysergide (5 mg/kg) and cyproheptadine (10 mg/kg). The behaviour was mimicked by the proposed 5-HT agonists lysergic acid diethylamide (0.1 and 0.2 mg/kg), 5-methoxy-N,N-dimethyltryptamine (0.5–1 mg/kg) and quipazine (1.5–10 mg/kg). Similarly treatment of rats with the 5-HT precursor L-tryptophan (25–50 mg/kg) together with the monoamine oxidase inhibitor pargyline (25 mg/kg) induced WDS.

A number of drugs thought to selectively interact with central catecholamine and cholinergic mechanisms were tested on the 5-HTP induced WDS response in rats. Only amphetamine (4 mg/kg, i.p.) and apomorphine (0.5 mg/kg, s.c.) produced significant inhibition of the response, with accompanying patterns of stereotyped behaviours.

A regional analysis of 5-HT concentrations in the brain showed that increases in this neurotransmitter following 5-HTP pretreatment paralleled the intensity of the WDS response. However, no clear differences in 5-HT levels in different brain regions were seen.

Bilateral electrolesions of the globus pallidus or of the lateral part of the ventral nucleus of the thalamus failed to modify the 5-HTP induced WDS. Similarly, frontal section at the level of the anterior commissure did not affect the response, while mid-diencephalic sections decreased it. WDS behaviour following 5-HTP was almost completely abolished by sections at the level of the posterior commissure.

We propose that the WDS may constitute a useful animal model for quantifying 5-HT activity in the central nervous system and screening potential agonists and antagonists of certain types of 5-HT receptors. It is probable that the WDS in the rat is closely related to the head twitches in mice (Corne, Pickering & Warner, 1963) and myoclonus in guinea-pigs (Klawans, Goetz, Westheimer & Weiner, 1973), while the hyperactivity syndrome (Grahame Smith, 1971) appears different. Hopefully these various models will help us distinguish possibly distinct 5-HT receptors in the CNS.

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## Pharmacological validation of a new test for the detection of antidepressant activity of drugs

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We have previously reported that bilateral ablation of the olfactory bulbs of rats results in diverse behavioural changes, and that most of these are reversed by chronic pretreatment with antidepressant drugs (Van Riezen, Schnieden & Wren, 1976). Amongst effective antidepressant drugs was mianserin. This drug was a false negative in conventional animal screening tests (Van Riezen, 1972) but has been reported to be an antidepressant in the clinical situation (Murphy, 1975; Wheatley, 1975). In particular, the 'anxiosoif' test has proved bery useful in assessing behavioural changes of bulbectomized rats which are sensitive to reversal by antidepressant drugs (Wren, Van Riezen & Rigter, 1976). We have now examined the specificity of this test for antidepressants by challenging it with a variety of psychoactive drugs.

The anxiosoif test is essentially a passive avoidance paradigm in which thirsty rats learn to avoid an electrified water spout. The apparatus consists of a rectangular cage, divided into two compartments by a clear perspex lid on one half and a black lid on the other. The spout of a water bottle protrudes through the black lid. The spout and the grid floor are connected to a stimulator delivering constant pulses of 1.0 mA.

In each experiment 60 male Wistar rats, weighing 170–220 g, were used. The animals were anaesthetized with tribromoethanol; 2.5 mg/kg i.p. Three groups of 10 rats were subjected to olfactory bulbectomy by means of bilateral aspiration, and 3 groups of 10 rats received sham-operation. After surgery the rats were allowed to recover for two weeks. Intraperitoneal drug treatment began on day 14 and continued daily throughout the testing period which commenced on day 21. One group of bulbectomized rats and one group of sham-operated rats received placebo treatment and the remaining groups were treated with a single dose of a test compound. The drugs and daily dosages used were amitriptyline (10 mg/kg), mianserin (10 mg/kg), imipramine (10 mg/kg), chlorimipramine (10 mg/kg), chlordiazepoxide (7.5 mg/kg), diazepam (5 mg/kg), chlorpromazine (1 mg/kg), haloperidol (0.05 mg/kg), (*dl*-8-chloro-11-antiamino-benzo-(b)-bicyclo 3.3.1 nona-3, 6a (10a) diene hydrochloride) (Org 6582) (10 mg/kg), and apomorphine (0.5 mg/kg).

The rats were maintained on a 23.5 h water deprivation schedule during the 4 testing days. They were allowed to drink in the apparatus for 10 min on day 1 and 2 of the test to obtain a measure of stable base-line levels of water intake. The procedure was repeated on day 3 and 4 except that the spout was electrified at every twentieth lick. The number of licks and shocks were recorded.

Removal of the olfactory bulbs tended to produce an increase in base-line levels of drinking in most experiments. With the exception of mianserin, antidepressant drugs (amitriptyline, imipramine, chlorimipramine) did not reverse this increase. The delivery of shock at every twentieth lick on day 3 and 4 led to a decrease in drinking behaviour in sham-operated animals. Water intake was even further decreased after treatment with Org 6582. The anxiolytic drugs chlordiazepoxide and diazepam attenuated passive avoidance behaviour in sham-operated rats, i.e. the shock-produced reduction in water intake was less pronounced in these animals.

Bulbectomized rats showed deficient avoidance behaviour: placebo-treated operated rats took more shocks than the corresponding sham-operated rats on day 3 as well as day four. This effect of the operation was selectively reversed by treatment with the antidepressants amitriptyline, imipramine, mianserin and chlorimipramine. A similar effect was obtained with Org 6582. Org 6582 was used in this investigation since it is a more potent and more selective inhibitor of 5-hydroxytryptamine re-uptake than chlorimipramine (Goodlet, Mireylees & Sugrue, 1976). Chlordiazepoxide, diazepam and chlorpromazine aggravated the deficiency in avoidance behaviour of bulbectomized rats. Apomorphine was virtually ineffective.

These results indicate that the alterations in passive avoidance behaviour induced in rats by the ablation of the olfactory bulbs can be reversed by antidepressants in distinction to anxiolytic and neuroleptic drugs. These data further strengthen the view that some of the behavioural changes which are seen in bulbectomized rats may serve as an animal model for the detection of antidepressant activity of drugs.

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# Effect of metoprolol and oxprenolol on delayed differentiation behaviour in the monkey (*Macaca mulatta*)

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In previous studies we have used a delayed differentiation task to study the behavioural effects of drugs (Nicholson, Wright & Ferres, 1973; Nicholson & Wright, 1974, 1976). The task involves the recognition of like or unlike visual stimuli separated by a few seconds, and the monkey is required to press a lever if the stimuli are like and to refrain from pressing a lever if the stimuli are unlike. Delayed differentiation has proved useful in the analysis of the activity of barbiturates and benzodiazepines, and so we have used the task to study $\beta$-adrenoceptor antagonists which may also have central effects (Leszkovszky & Tardos, 1965; Bainbridge & Greenwood, 1971).

Five male monkeys (*Macaca mulatta*) of mean body weight 11.6 kg were used. On separate occasions each monkey was injected intraperitoneally with 5, 10, 15, 20, 25 or 30 mg/kg metoprolol or oxprenolol. The drug vehicle alone (saline) was injected on four occasions. A random order of injection was used, and each injection was separated by at least four days.

Performance was tested 1 and 4 h after injection, though with 30 mg/kg oxprenolol the monkeys failed to respond to or to complete the task at 1 hour. The data were analysed by analysis of variance.

Total response time was increased at 1 h with 20 and 25 mg/kg oxprenolol ($P < 0.05$ and 0.001 respectively) and with 20, 25 and 30 mg/kg metoprolol ($P < 0.01$, 0.001 and 0.001 respectively). Quadratic equations were fitted to the data for each drug, but, as the equations did not differ, the data were combined. The curve was forced through the origin to give the relation for increase in total response time with dose as $0.176\ (\text{mg/kg})^2 - 1.63\ (\text{mg/kg})$. Total response times at 4 h were also increased, and this effect was observed with all doses above 15 mg/kg metoprolol and 20 mg/kg oxprenolol ($P < 0.01$).

Analysis of accuracy of response was carried out by combining the data for 5 and 10 mg/kg, 15 and 20 mg/kg and 25 and 30 mg/kg. At 1 h accuracy of response was impaired with 5 and 10 mg/kg ($P < 0.01$) and 15 and 20 mg/kg ($P < 0.01$) oxprenolol, and with 15 and 20 mg/kg ($P < 0.05$) and 25 and 30 mg/kg ($P < 0.001$) metoprolol. At 4 h accuracy of response was impaired with 25 and 30 mg/kg oxprenolol ($P < 0.001$), but with metoprolol the effect was observed throughout the dose range ($P < 0.01$), even though with 5–10 mg/kg no effect was observed 1 h after injection.

These results suggest that the $\beta$-adrenoceptor antagonists modify delayed differentiation in a way different from that observed with barbiturates and benzodiazepines. With barbiturates and benzodiazepines impaired differentiation is observed only when total response time is increased, but with metoprolol and oxprenolol accuracy of response may be impaired without prolongation of the total response time.

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## Cocaine and amphetamine as discriminative stimuli in rats

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Amphetamine can serve as a discriminative stimulus since rats can be trained to make different behavioural responses depending on whether they have received the drug or saline. Little is known about the discriminative stimulus properties of cocaine, which has therefore been compared directly with amphetamine using the procedure described by Kuhn, Appel and Greenberg, 1974. Initially, rats were trained to press bars for water reinforcement; a tandem schedule of reinforcement was used in which, after a variable interval of time (mean = 1 min) in which bar-pressing had no consequences, the 10th bar-press was reinforced. Half of the rats were then reinforced for pressing the left bar when drugged and the right bar after saline. To balance out position preferences, these contingencies were reversed in the remaining rats. Four rats were trained with (+)-amphetamine sulphate (1.0 mg/kg, i.p.) injected 30 min before 30 min training sessions. Another 4 rats were trained with cocaine hydrochloride (10.0 mg/kg, i.p.) injected 15 min before sessions.

Amphetamine, but not cocaine, increased the overall rate of responding by about 32% ($P < 0.05$); however, choice of the correct bar developed at about the same rate for both drugs. After 30–35 training sessions, discriminative control by the drugs was confirmed in brief (5 min) test sessions in which no responses were reinforced. Responding on the bar previously appropriate for amphetamine was $91.4 \pm 5.3\%$ after amphetamine, as compared with $6.4 \pm 4.9\%$ after saline (means ± s.e. mean $P < 0.01$). The corresponding results with cocaine were $85.6 \pm 6.9\%$ and $5.4 \pm 1.5\%$ ($P < 0.01$).

Dose-response curves were then established by injecting various doses of amphetamine or cocaine in balanced sequences before test sessions. $ED_{50}$ values were defined as doses producing 50% responding on the bar appropriate for drug. The $ED_{50}$ was $0.30 \pm 0.07$ mg/kg for amphetamine and $2.8 \pm 0.4$ mg/kg for cocaine (means ± s.e. mean). The dose-response curves were then determined again but this time, amphetamine was administered to the rats trained with cocaine, and *vice versa* (crossover phase). In adequate doses, both amphetamine and cocaine produced responding on the bar appropriate for the training drug, suggesting a close similarity between their discriminative stimulus properties. However the $ED_{50}$ values were now $0.62 \pm 0.18$ mg/kg for amphetamine and $10.1 \pm 2.7$ mg/kg for cocaine, significantly higher than in the first phase of the experiment ($P < 0.05$). The changes in potency can be accounted for either by the development of tolerance or by differences in the discriminative effects of the two drugs. Generally, it is very difficult to develop tolerance to the discriminative effects of drugs (Jones, Grant & Vospalek, 1976; York & Winter, 1975). The present results therefore indicate the possible importance of complete cross-over designs in combination with $ED_{50}$ determinations when attempting to classify drugs according to their discriminative properties.

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## Influence of morphine dependence and withdrawal on circling behaviour in rats with unilateral nigral lesions

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Changes in brain dopamine may be involved in certain behavioural manifestations of morphine dependence and abstinence (Gianutsos, Hynes, Puri, Drawbaugh & Lal, 1974; Kuschinsky & Hornykiewicz, 1974; Iwamoto, Loh & Way, 1976; Lal, 1975). One possibility is that dopamine receptors become 'supersensitive' after chronic morphine administration. We report some behavioural tests of striatal dopamine receptor sensitivity during different stages of morphine dependence.

Male hooded rats with unilateral electrolytic lesions of the substantia nigra were used; the co-ordinates of the lesions were A: 2.8; L: 2.3; V: 2.5 (De Groot, 1959). Rats ($n = 8$) were selected from a pool of animals displaying 'circling' behaviour ipsilateral to the side of lesioning after doses of apomorphine, a direct dopamine agonist. Circling behaviour was recorded automatically, the number of whole rotations in each direction being accumulated and printed periodically.

The first set of experiments examined whether the base-line response to apomorphine remained stable over time. Three dose-response curves were therefore obtained at intervals of one month. Doses of apomorphine HCl in these and all subsequent tests were 0, 0.25, 0.5 and 1.0 mg/kg, s.c., each rat receiving every dose according to a Latin-square design with an interval of 2–3 days between doses. Circling behaviour was recorded for 90 min after injection with apomorphine. The drug induced ipsilateral turning in a dose-related manner and there was no significant difference between the first curve and its two replications ($F = 1.98$, d.f. 2,77).

The rats were then injected daily with morphine HCl (10 mg/kg, i.p.) for two weeks and their circling response to apomorphine was recorded both when they were in a state of 23 h withdrawal and also starting 60–90 min after the injection of morphine. Withdrawal from this dose of morphine significantly increased apomorphine-induced circling behaviour ($F = 9.69$, d.f. 1,49, $P < 0.01$—the analysis of variance comparisons here and subsequently refer to the final baseline curve obtained with apomorphine). Testing in the presence of morphine also resulted in increased circling ($F = 7.24$, d.f. 1,35, $P < 0.01$).

The dose of morphine was increased and then maintained at 100 mg $kg^{-1}$ $day^{-1}$ for the next 22 days. The rats were retested with apomorphine as before, i.e. in withdrawal 23 h or 60–90 min after morphine. There was a further increase in circling behaviour both in withdrawal ($F = 20.03$, d.f. 1,49, $P < 0.001$) and in the presence of morphine ($F = 24.77$, d.f. 1,35, $P < 0.0001$). The nature of increased circling was, however, different in the treated and withdrawn states. In withdrawal there was an increase in the slopes of the dose-response curves. The difference between the slopes in withdrawal from 100 mg/kg morphine ($86.69 \pm 19.98$ turns/log unit dose) and control ($38.69 \pm 8.56$ turns/log unit dose) was significant ($t = 2.21$, d.f. 21, $P < 0.05$). In contrast, the slope values obtained in the presence of 10 and 100 mg/kg morphine were not changed; they were $41.94 \pm 9.06$ and $36.81 \pm 14.08$ turns/log unit dose respectively. In dependent rats morphine by itself induced ipsilateral circling. Thus, in the presence of morphine an apparent increase in the sensitivity to apomorphine reflected the summation of an unchanged net response to apomorphine with a dose-related increase in circling due to morphine.

The enhanced response to apomorphine in morphine abstinence appears to be related to the level of dependence and it may reflect a change in the sensitivity of one or more populations of dopamine receptors.

This work was supported by a grant from the M.R.C.

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## Effect of a potent synthetic opioid pentapeptide in some anti-nociceptive and behavioural tests in mice and rats

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The anti-nociceptive action of the two endogenous morphine-like pentapeptides, methionine- and leucine-enkephalin (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975) is extremely weak and transient and is only apparent after injection into the brain (Büscher, Hill, Römer, Cardinaux, Closse, Hauser & Pless, 1976; Belluzzi, Grant, Garsky, Sarantakis, Wise & Stein, 1976). Synthetic pentapeptides with increased opiate receptor affinity (Chang, Fong, Pert & Pert, 1976) and with a longer duration of anti-nociceptive action have been reported (Pert, 1976). A potent synthetic opioid pentapeptide analogue of leucine-enkephalin has recently been found having potency ratios relative to morphine of 1227 in the isolated mouse vas deferens and 1.95 in the isolated guinea-pig ileum preparation (Beddell, Clark, Hardy, Lowe, Ubatuba, Wilkinson, Miller, Chang & Cuatrecasas, unpublished observations). We have now undertaken some comparative studies on this peptide, Tyr.D-Ala.Gly.Phe.D-Leu (BW 180C) and morphine injected intracerebroventricularly (i.c.v.) in some behavioural and antinociceptive studies in mice and rats.

Anti-nociceptive studies were undertaken in mice using standard stimuli: thermal (hot plate at 55°C), mechanical (tail clip) and chemical (phenylbenzoquinone induced writhing). The $ED_{50}$ values (µg/mouse) at 20 min after drug in the three tests were respectively 0.10, 0.07 and 0.007 µg for BW 180C and 0.33, 0.21 and 0.015 µg for morphine. The anti-nociceptive action of the peptide was of shorter duration than that of morphine. Simultaneous treatment with the specific morphine antagonist naloxone (78 µg/kg, s.c.) produced a parallel shift of the dose-response curves for both morphine and the peptide in the hot-plate test.

Behavioural and locomotor activity studies in mice (5 or 6 groups, $n = 4$, per treatment) revealed that both the peptide and morphine (i.c.v.) increased locomotor activity as measured by ultrasonic activity monitoring equipment (6 channel; C.F. Palmer, High Wycombe). Activity was significantly increased ($P \leqslant 0.01$) by the peptide at doses $\geqslant 0.04$ µg and by morphine at $\geqslant 0.3$ µg. The effect of the peptide was less persistent than that of morphine. The hyperactivity induced by the peptide in mice may reflect an increased dopaminergic transmission as has been suggested for morphine (Kuschinsky, 1976). This suggestion is supported by the finding that the known dopamine antagonist, chlorpromazine (5 mg/kg, i.p.) given 1 h previously abolished the increased activity induced by the peptide (0.15 μg) and by morphine (0.6 μg).

The effect of the peptide (i.c.v.) on the EEG of conscious rats was studied in animals (male 300 to 350 g) chronically implanted with skull electrodes and intraventricular cannula (Goff, Miller, Smith, Smith & Wheatley, 1975). The peptide at 5 μg ($n=2$) induced EEG spiking of almost immediate onset followed by facial and forelimb clonus 6 min later. Spiking continued for at least 45 min but the clonus was of shorter duration (9 and 30 min respectively for each rat). Morphine is known to induce EEG spiking when injected directly into the brain of rats (Teitelbaum, Blosser & Catravas, 1976).

Our studies on BW 180C provide further evidence that synthetic opioid pentapeptides which are less labile than endogenous enkephalins possess an antinociceptive action similar to that of morphine (Pert, 1976). Additionally, our studies have revealed that these peptides may also possess a similar behavioural profile to morphine.

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## Reversal of morphine-induced suppression of active avoidance behaviour by the tetracyclic antidepressant mianserin

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The antidepressant drug mianserin (Org GB 94) has been shown to effectively antagonize in rats the catatonia (both muscle rigidity and akinesia) produced by morphine (Preston, unpublished observations). The present study was undertaken to examine if mianserin would also antagonize other behavioural effects of morphine. It has been reported that morphine depresses avoidance responses in rats (Verhave, Owen & Robbins, 1959). We have therefore tested whether mianserin and some other antidepressants or 5-hydroxytryptamine (5-HT)-receptor blocking agents antagonize the incapacitating effect of morphine on the performance of a two-way active avoidance response.

For each experiment 50 or 60 male Wistar rats (weighing 200–250 g) received avoidance training in automated shuttleboxes. During a trial a light in the lid of the box signalled impending electric shock to the feet through the grid floor. A rat could prevent onset of the shock by moving to the opposite side of the box within the first 6 s of illumination (avoidance). Shuttling after the onset of shock caused the current to shut off (escape); failure to escape led to 25 s of shock. Trials took place once a minute. In addition to the number of avoidances and escapes, the number of intertrial responses was also recorded.

The training schedule was as follows: days 1, 2 and 3: 50 trials per day; day 4: 25 trials. On day 5, rats were randomly allotted to groups (9–10 rats per group) which received either placebo s.c. + placebo i.p., 10 mg/kg morphine s.c. + placebo i.p., or 10 mg/kg morphine s.c. + test drug i.p. All injections were given 40 min before the start of the test session of 25 trials. The compounds studied were mianserin (4 and 16 mg/kg), amitriptyline (1, 4 and 16 mg/kg), imipramine (1, 4 and 16 mg/kg), methysergide (2.5 and 5 mg/kg) and cinanserin (4 and 16 mg/kg). The two-tailed permutation test was used for the statistical evaluation of the results.

Rats rapidly acquired the avoidance response. Treatment with morphine not only eliminated avoidance responses but also strongly suppressed escape and intertrial responses. Mianserin only slightly reversed the effect of morphine on avoidance behaviour. However, rats given morphine + mianserin showed a significantly larger number of escapes and intertrial responses than morphine + placebo-treated animals. Methysergide and cinanserin also restored the ability of morphine-treated rats to score escape and intertrial responses, although to a lesser degree than mianserin. Amitriptyline and imipramine were ineffective.

Methysergide and cinanserin have been shown to antagonize the antinociceptive action of intracerebrally administered morphine (Yaksh, DuChateau & Rudy, 1976). Like methysergide and cinanserin, mianserin possesses 5-HT receptor blocking properties (Vargaftig, Coignet, de Vos, Grijsen & Bonta, 1971). The ability of these compounds to antagonize the incapacitating effect of morphine on the performance of rats in a shuttlebox supports the idea that 5-HT mechanisms are involved in some of the effects of morphine. The restoration of intertrial responses by mianserin, methysergide and cinanserin suggests that these drugs reversed the morphine-induced akinesia. Restoration of escape responses indicates that antagonism of analgesia may also be involved.

Mianserin, though a clinically effective antidepressant (Murphy, 1975; Wheatley, 1975), was a negative in conventional animal screening tests for antidepressants (Van Riezen, 1972). The finding that amitriptyline and imipramine failed to act like mianserin in the present experiments adds to the notion that the latter compound may be pharmacologically different from tricyclic antidepressants.

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## The effect of ethanol on a passive avoidance task in rats

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It has previously been shown (Chesher, 1974) that ethanol (1.5 g/kg, i.p.) enhances the learning of an active conditioned avoidance task in rats. This effect was abolished if the animals had been pre-treated with the catecholamine synthesis inhibitor $\alpha$-methyl-$p$-tyrosine. The task made use of a two-compartment shuttle box; to escape an electric shock, the animals had to learn to change compartments within 5 s of the sounding of a buzzer.

In the experiments to be described the effect of ethanol was tested on a passive avoidance task: instead of running to another compartment, the animals had to refrain from doing so to avoid a shock.

The apparatus, similar to that described by Jarvik & Kopp (1967), consisted of a two-compartment box with an interconnecting opening. The rat was placed into one compartment which was lit by a 25 W incandescent light, and the time before it entered the adjacent, dark compartment was determined. Rats usually prefer the dark when given a choice. A shutter prevented the rat from retracing its steps, and it was given a footshock of 1 mA for 5 seconds. Animals were re-tested in the same apparatus 1 or 7 days later. Male Sprague-Dawley rats (200–390 g) were used.

When tested 30 min after ethanol (1.5 g/kg, i.p.) rats ($n = 45$ per group) entered the dark compartment on both trial 1 and trial 2 with a significantly shorter latency than saline controls (trial 1, saline mean latency 12 ± 1 s, ethanol 7 ± 1 s; trial 2, saline 137 ± 11 s, ethanol 23 ± 7 s; $P < 0.001$). Dose response relations could be demonstrated in both trials for doses of ethanol between 0.9 and 2.0 g/kg ($n = 15$ or 20 per group). If rats were not shocked in the dark compartment at trial 1, latencies at trial 2 did not differ significantly from trial 1. Pre-treatment with $\alpha$-methyl-*p*-tyrosine (80 mg/kg, i.p.) 3.5 h before ethanol or saline did not affect the ethanol induced changes at either trial ($n = 17$ per group).

The test procedures in both kinds of experiment described depended on the initiation of movement. In the active avoidance task ethanol led to quicker learning, and this effect was abolished by pre-treatment with $\alpha$-methyl-*p*-tyrosine, which suggests that a newly synthethized catecholamine may have been involved. In the passive avoidance task ethanol hindered learning and this was not affected by $\alpha$-methyl-*p*-tyrosine.

Although the effect of ethanol on the performance of both these tasks in rats can be described as 'stimulating' movement, each is presumably mediated by different neuronal pathways.

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## Effect of iontrophoretic and intravenously administered atropine on the acetylcholine-discharges of lateral geniculate neurones

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In the present study, acetylcholine (ACh) sensitivity of lateral geniculate cells was re-examined after iontophoretically and intravenously administered atropine under different experimental conditions.

Conventional techniques for extracellular recording with 5-barrel micropipettes and iontophoretic drug application were used (Krnjević & Phillis, 1963). The outer barrels were filled with the following substances: ACh (1 M, pH 4.9), atropine sulphate (10 mM in 165 nM NaCl, pH 5.5), mecamylamine HCl (10 mM in 165 mM NaCl, pH 6.6) and L-glutamate (1 M, pH 7–7.4). Geniculate neurones of lamina A were identified by the visual evoked response and by the neuronal sensitivity to L-glutamate and ACh. Subsequently, dye deposition was achieved to mark recording sites and to permit the histological reconstruction of electrode tracks (Godfraind, 1969, 1976; Godfraind & Meulders, 1969). All animals, except one, received an injection of atropine methylnitrate (4 mg/kg, i.v.), and were paralyzed with an intravenous infusion of succinylcholine at an approximate rate of 1 mg/min.

In an anaesthetized cat (fluothane 1%), and in a non-anaesthetized midpontine pretrigeminal preparation, the observations made were in agreement with previous descriptions: neuronal discharges induced by ACh were depressed either by atropine or by mecamylamine applied by iontophoresis, as well as after an intravenous injection of atropine sulphate (3 mg/kg) (Curtis, 1966; Curtis & Crawford, 1969; Marshall & McLennan, 1972).

Different results were observed under $\alpha$-chloralose (80 mg/kg, i.v.; 3 cats) and urethane (1 g/kg, i.v.; 2 cats) anaesthesia. Under these conditions, discharges evoked by ACh (15–45 nA, 10–20 s) were also prevented or greatly reduced by iontophoresis of either atropine (10–20 nA, 40–50 s) or mecamylamine (10–30 nA, 30–65 s). However, after atropine sulfate i.v. administration (3 mg/kg), neuronal discharges could still be induced by ACh applied with similar iontophoretic parameters. Tests with cholinergic antagonists applied by iontophoresis were again performed on the same units to analyse the pharmacology of these 'remaining' ACh responses. These appeared to be more resistant to iontophoretically applied atropine than before the i.v. atropine sulphate injection. Indeed, after atropine had been iontophoretically reapplied with the same parameters, some of the ACh responses were comparable to the control, while others were slightly delayed by about 3 to 5 s. On the contrary, mecamylamine was a good antagonist: 7 to 30 nA mecamylamine for about 30–65 s was sufficient to depress ACh induced activity (20–45 nA ACh for 10–20 s). This antagonism was shown to be reversible provided a small dose of mecamylamine was applied.

In conclusion, the present results indicate that α-chloralose or urethane anaesthesia combined with the intravenous administration of atropine sulphate, provide favourable experimental conditions for the study, in the thalamus, of ACh responses which might be attributed to the stimulation of nicotinic receptors.

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# The effect of cholinomimetic drugs on the isolated hemisected immature rat spinal cord

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Motoneuronal responses of the isolated hemisected immature rat spinal cord (Otsuka & Konishi, 1974) were measured as changes in ventral root polarity. Drugs were dissolved in 2 ml Ringer solution and perfused on to the isolated hemicord at 0.8 ml/minute. The temperature of the preparation was maintained at 20 ± 0.5°C.

Acetylcholine (1–10 mM) evoked depolarizing responses whereas choline (1–10 mM) had no effect. Continuous perfusion with neostigmine (1 µM) produced a small depolarization which levelled off and at this stage depolarizing responses to 10–100 µM acetylcholine could be obtained.

It was surprising to find that responses to acetylcholine were still elicited from hemicords treated with 1 mM procaine or 0.1 µM tetrodotoxin; an indication that motoneurones were responding to cholinomimetics directly.

Responses to 50 µM acetylcholine were abolished by atropine (1 µM) and were unaffected by tubocurarine (10 µM) or hexamethonium (10 µM). Thus is would appear that motoneurones of the immature rat possess muscarinic receptors. Atropine sensitive acetylcholine responses of cat motoneurones *in vivo* have been demonstrated recently by Zieglgänsberger and Bayerl (1976).

This work was supported by the Medical Research Council.

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# Effects of viloxazine on cortical neurone responses to monoamines and acetylcholine

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Viloxazine hydrochloride (ICI 58,834; Vivalan, ICI Ltd.) is a substance belonging to a series of novel psychotropic compounds (Grrenwood, Mallion, Todd & Turner, 1975) and has been found to be a clinically effective antidepressant drug (Peet, 1973; Pichot, 1975). Previous pharmacological studies indicate that viloxazine inhibits the neuronal uptake of noradrenaline (NA) and to a much lesser extent 5-HT but, in contrast to tricyclic antidepressants, it possesses little or no peripheral cholinolytic activity (Greenwood, 1975; Lippmann & Pugsley, 1976). We have used the technique of microiontophoresis to investigate the interactions of this drug with NA, 5-HT and acetylcholine (ACh) on the spontaneous activity of cortical neurones.

Single spontaneously active neurones were studied in the somatosensory cortex of the halothane anaesthetized rat. The techniques used have been described elsewhere (Bradshaw, Roberts & Szabadi, 1974). Repeated responses to NA, 5-HT and Ach were compared before and after the brief application of viloxazine (20–100 nA, 20–50 s).

Following the application of viloxazine, responses to all three agonists were either potentiated or reduced in size. Both effects often occurred in the same study, reduction of response invariably preceding potentiation. The time course for these effects typically showed reduction of response occurring during the first 5–10 min after viloxazine application followed by potentiation of response over the next 15–40 minutes. These effects are similar to those reported for imipramine, desipramine and iprindole where reduction of response followed high doses and potentiation low doses of antidepressant (Bradshaw *et al.*, 1974; Bevan, Bradshaw & Szabadi, 1975a, b).

Since viloxazine can block amine uptake mechanisms (Greenwood, 1975; Lippmann & Pugsley, 1976) the potentiation of NA and 5-HT responses can be explained. The reduction of NA and 5-HT responses possibly indicates that viloxazine in common with tricyclics possesses adrenolytic and anti-5-HT properties.

The effects on Ach responses were less explicable. Bevan *et al.* (1975a) explained the potentiation of Ach responses by impramine and desipramine in terms of the postsynaptic blockade of 'masked' inhibitory receptors. This would not seem to be a satisfactory explanation for the potentiation of Ach responses by viloxazine since it has been reported to have little or no cholinolytic activity. However, since ACh responses of single cells could be reduced by viloxazine, it may be that CNS receptors for Ach are more susceptible to blockade by viloxazine than those studied in the periphery and it is of interest that viloxazine antagonizes oxotremorine-induced hypothemia but not tremor or salivation (Lippmann & Pugsley, 1976).

An action of viloxazine on the inactivation of Ach would also seem possible, i.e. cholinesterase activity may be reduced or the re-uptake process for Ach proposed by Liang and Quastel (1969) may be blocked. There is no evidence, however, that viloxazine has either of these actions.

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# Role of tryptaminergic mechanisms in the elements of the behavioural syndrome evoked by tryptophan and a monoamine oxidase inhibitor

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When treated with tryptophan and a monoamine oxidase inhibitor (MAOI), rats develop a syndrome of hyperactivity, hyperreactivity, reciprocal forepaw treading, head weaving, and hind-limb abduction (Hess & Doepfner, 1961). The same syndrome can be produced by the 5-HT releasing drug, *p*-chloroamphetamine. The hyperactivity component of the syndrome is abolished by pre-treatment with *p*-chloro-phenylalanine (Grahame-Smith, 1971). Changes in automatically-recorded activity following tryptophan and MAOI have been used as an index of 5-HT receptor activation, and the interaction of a variety of agents with tryptaminergic mechanisms has been investigated in this way (Green & Grahame-Smith, 1976). Jacobs (1974) has investigated the effects of various treatments on ratings of the individual features of the syndrome, but the results of the two approaches do not always agree. For example, Green & Grahame-Smith (1976) are led by their findings to postulate a dopaminergic link in the production of the syndrome, whereas Jacobs (1974) concludes that the whole syndrome is tryptaminergic.

We have investigated the effects of a 5-HT receptor blocking drug, methergoline (Fuxe, Agnati & Everitt, 1975), on the syndrome using both automated recordings of the hyperactivity and ratings of the individual components of the syndrome. Following the procedure of Grahame-Smith, animals were caged in groups of three, and locomotor activity was automatically recorded with an Animex apparatus. Tryptophan (50 or 100 mg/kg) was administered 30 min after tranylcypromine (10 or 20 mg/kg). *p*-Chloroamphetamine was used to elicit the syndrome in a dose of 10 mg/kg. Methergoline was administered in doses of 2 and 5 mg/kg at the same time as tranylcypromine or 30 min before *p*-chloroamphetamine. Ratings of head weaving, forepaw treading, hind-limb abduction and other components of the syndrome were made by an observer at intervals after drug administration.

Methergoline abolished forepaw treading, head weaving and hind-limb abduction whether elicited by *p*-chloroamphetamine or by tranylcypromine and tryptophan. This striking effect was not paralleled by abolition of the increased activity in either the *p*-chloroamphetamine or tranylcypromine and tryptophan groups. On the contrary, in some groups of animals in which head weaving and forepaw treading had been prevented by methergoline pre-treatment, increased and well-coordinated locomotor activity was observed. Methergoline alone was without effect on locomotor activity.

In summary, a marked qualitative change induced in the tryptophan-MAOI syndrome by 5-HT receptor blockade was not reflected in automated recordings of locomotor activity. This raises doubts as to whether changes in locomotor activity necessarily reflect changes in central tryptaminergic transmission. The results also suggest that different components of the syndrome may have different neurochemical bases.

J.F.W.D. is a Medical Research Council Fellow.

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# Precursors of 5-hydroxytryptamine reduce the pressor response to clonidine in the rat

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Intravenous injection of clonidine causes a brief pressor response which precedes its hypotensive action (Zaimis, 1970). We have now found that the magnitude of the pressor effect can be markedly reduced by prior administration of either L-tryptophan or L-5-hydroxytryptophan (5-HTP).

Male hooded unanaesthetized rats (200–300 g) were initially used, arterial blood pressure and heart rate in each being directly recorded. Clonidine injected intravenously caused a rise in blood pressure lasting approximately 15 min, and bradycardia. The pressor response was significantly smaller in groups pretreated with either tryptophan or 5-HTP and this inhibition was prevented by prior administration of Ro4-4602 (Benserazide) or methysergide (Table 1).

In anaesthetized rats (700 mg/kg urethane i.p.), tryptophan (100 mg/kg, s.c.) given 90 min before, caused a parallel shift to the right (2.2 fold) of the regression line relating mean rise in blood pressure to log intravenous dose of clonidine hydrochloride (16–64 μg/kg). Analogous curves for (−)-adrenaline bitartrate (2–8 μg/kg) or angiotensin II amide (0.16–4.0 μg/kg) were not significantly changed. The inhibitory effect of tryptophan was reduced by pithing, Ro4-4602 (200 mg/kg, i.p.), carbidopa (100 mg/kg, i.p.) and methysergide maleate (1.5 mg/kg, i.p.) given respectively 60–120 min, 120 min and 30–120 min before clonidine.

The magnitude of clonidine's pressor effect depends on the equilibrium established between the rise in arterial pressure due to vasoconstriction and opposing depressor influences. The latter include reflex

**Table 1** Mean changes (±s.e. mean) in blood pressure and heart rate caused by clonidine hydrochloride (25 μg/kg) intravenously in rats pretreated with drugs affecting tryptaminergic mechanisms

| *Pretreatment (doses in parentheses)* | *Time (min) administered before clonidine* | *Route* | *No. of animals* | *Mean BP (mmHg)* | *Mean rise in BP (mmHg) due to clonidine* | *Mean HR (beats/min)* | *Mean Bradycardia (beats/min)* |
|---|---|---|---|---|---|---|---|
| 1. Saline controls | 90 | S.C. | 25 | 119±2 | 27±2 | 366±11 | 80±9 |
| 2. L-tryptophan (100 mg/kg) | 90 | S.C. | 9 | 113±4 | 4±6* | 390±25 | 60±16 |
| 3. 5-hydroxytryptophan (100 mg/kg) | 90 | S.C. | 7 | 86±5* | 3±1* | 393±8 | 9±8 * |
| 4. Ro4-4602 (2×100 mg/kg) | 180<br>60 | I.P. | 7 | 123±5 | 32±1 | 332±17 | 75±21 |
| 5. L-tryptophan (100 mg/kg) | 90 | S.C. | 7 | 119±3 | 29±3 | 332±18 | 90±14 |
| + | | | | | | | |
| Ro4-460– (2×100 mg/kg) | 180<br>60 | I.P. | | | | | |
| 6. Methysergide maleate (3×0.5 mg/kg) | 120<br>60<br>30 | I.P. | 6 | 104±2* | 48±6* | 278±14* | 38±9* |
| 7. L-tryptophan (100 mg/kg) | 90 | S.C. | 6 | 99±5* | 46±7* | 284±9* | 53±6 |
| + | | | | | | | |
| Methysergide maleate (3×0.5 mg/kg) | 120<br>60<br>30 | I.P. | | | | | |

* Significantly different from mean response of saline controls ($P < 0.05$, Analysis of variance, F-test)

barostatic mechanisms and depression by the drug of bulbar efferent sympathetic tone (Schmitt, Schmitt, Boissier & Giudicelli, 1967) and peripheral adrenergic nerve function (Boura, 1975). Present results suggest that the point of this equilibrium can be specifically affected by tissue levels of 5-hydroxytryptamine. That this effect was mediated by a peripheral mechanism was suggested by the action of carbidopa, a selective inhibitor of peripheral L-aromatic amino acid decarboxylase (Bartholini & Pletscher, 1969) but the abolition of the inhibitory effect by pithing indicated that the central nervous system may also be involved.

This work was partly supported by a grant from The Wellcome Foundation Limited.

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## Responses of the hepatic arterial vascular bed of the dog to intra-arterial injections of noradrenaline, adrenaline and phenylephrine: the role of $\beta$-adrenoceptors

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In the sympathetically-denervated hepatic arterial vascular bed of the dog, intra-arterial (i.a.) injections of noradrenaline cause dose-dependent hepatic arterial vasoconstriction (Richardson & Withrington, 1976a). Whilst previous reports have suggested that the effect of adrenaline on this vascular bed is essentially vasoconstrictor (see Greenway & Stark, 1971), isoprenaline injected i.a. causes dose-dependent hepatic arterial vasodilatation, due to stimulation of $\beta_2$-adrenoceptors (Richardson & Withrington, 1976b). The contribution of $\beta$-adrenoceptor stimulation to the effects of the catecholamines has therefore been examined in the sympathetically-innervated hepatic arterial vasculature.

The hepatic arterial vascular beds of 15 chloralose-urethane anaesthetized dogs (13.1 ± 2.2: mean ± s.d. kg) were perfused as described by Richardson & Withrington (1976c): under control conditions, the hepatic arterial perfusion pressure was 117.7 ± 15.4 (s.d.) mmHg and the hepatic arterial blood flow 210.7 ± 56.9 ml/min. The calculated hepatic arterial vascular resistance (HAVR) was 1.80 ± 0.76 mmHg $ml^{-1}$ min 100 g, the livers weighing 301.6 ± 50.1 grams.

When noradrenaline was injected i.a., there was an immediate dose-dependent increase in the calculated HAVR at doses above 5 ng, with a maximum rise in HAVR of 308.5 ± 81.1% (mean ± s.e. mean; $n = 10$) occurring between 50 and 200 µg. In addition, over the dose range 50 ng–10 µg in most experiments, there followed a secondary reduction in HAVR which reached a maximum of 17.2 ± 3.4% and had a time course similar to that of i.a. injections of isoprenaline (see Richardson & Withrington, 1976c).

The responses to i.a. adrenaline were similar to those of noradrenaline, with dose-dependent rises in HAVR at doses above the threshold (5–100 ng) reaching a maximum of 504.5 ± 79.8% ($n = 11$) at 50–200 µg. These were followed by vasodilatation at doses between 50 ng and 50 µg reaching a maximum of 21.7 ± 3.3%. In all experiments where both catecholamines were administered, noradrenaline was on a molar basis a more potent vasoconstrictor than adrenaline.

Phenylephrine caused dose-dependent rises in HAVR above the threshold of 1 µg i.a., reaching a maximum of 412.9 ± 87.2% ($n = 4$) at 50–200 µg, but in contrast to noradrenaline and adrenaline, there was no secondary reduction in HAVR.

In 3 experiments, propranolol (0.25 mg $kg^{-1}$, i.v.) markedly attenuated the secondary dilatation due to i.a. noradrenaline and adrenaline. In addition, both catecholamines were more potent hepatic arterial vasoconstrictors; the increase in vasoconstrictor potency of adrenaline, but not of noradrenaline, being statistically significant (Paired $t$-test; $P < 0.02$). The mean dose of adrenaline which doubled the HAVR before propranolol was $1.7 \times 10^{-8}$ mol, and after propranolol $1.8 \times 10^{-9}$ mol (for noradrenaline: $3.9 \times 10^{-9}$ and $2.6 \times 10^{-9}$ mol respectively).

The vasoconstrictor potency of phenylephrine was unaffected by propranolol.

Whilst the predominant effects of noradrenaline and adrenaline on the hepatic arterial vasculature are vasoconstrictor, a secondary vasodilatation occurs with these catecholamines, but not with phenylephrine. The potentiation of the vasoconstriction and attenuation of the secondary vasodilatation due to the catecholamines by propranolol illustrates the role of $\beta$-adrenoceptor stimulation in the responses of this vascular bed to i.a. catecholamines.

We thank the Medical Research Council for financial support.

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# Turning behaviour elicited by apomorphine, (+)-amphetamine and three ergot derivatives in rats treated with a unilateral injection of ethanolamine-*O*-sulphate in the substantia nigra

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Recently, Dray, Oakley & Simmonds (1975) have reported that apomorphine and (+)-amphetamine elicit ipsilateral turning in rats which have received a unilateral injection of ethanolamine-*O*-sulphate (EOS) into the substantia nigra. After EOS injection, these animals showed an elevation of GABA concentration in the substantia nigra brought about by an inhibition of GABA transaminase (Fowler, 1973). This may correspond to an activation of an inhibitory GABA-minergic pathway from the striatum to the dopamine containing neurons of the zona reticulata of the substantia nigra (McGeer, McGeer, Wada & Jung, 1971; Kim, Bak, Hassler & Okada, 1971). We wish to report on the effects of three ergot derivatives which have been shown to elicit contralateral turning in rats with a unilateral degeneration of the nigro-striatal pathways after administration of 6-hydroxydopamine into the substantia nigra.

Under nembutal anaesthesia, male OFA rats (250 g) received unilateral injections of EOS (50 µg in 1.5 µl) stereotaxically directed into the zona reticulata of the substantia nigra. Drugs were administered s.c., 24 h after EOS, 6 rats per group. Behaviour was assessed during the following 7 hours. Injection sites were confirmed histologically.

As reported by Dray, Oakley & Simmonds (1975), apomorphine and (+)-amphetamine (1–4 mg/kg) both elicited ipsilateral turning. After 4 mg/kg, the total number of turns was $513 \pm 87$ and $852 \pm 111$, respectively. In contrast to the results of Dray, Fowler, Oakley, Simmonds & Tanner (1975), bromocriptine (10 or 50 mg/kg) elicited ipsilateral postural asymmetry and stereotyped sniffing. Ipsilateral turning was only observed upon handling. The ergoline derivative CF 25–397 (Jaton, Loew & Vigouret, 1976) induced only contralateral asymmetry at doses of 20 to 50 mg/kg. LSD–25 (1 or 4 mg/kg) induced typical contralateral turning. After 4 mg/kg, the total number of turns was $210 \pm 49$.

These results indicate that the three ergot derivatives investigated exert differential actions in EOS treated rats. As opposed to apomorphine and (+)-amphetamine, LSD–25 was the only compound which elicited contralateral turning after unilateral inhibition of GABA metabolism.

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# Specificity of monoamine neurotoxins: rotational responses to dopaminergic agonists after unilateral 6-OHDA and 5,6-DHT lesions of the median forebrain bundle

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The relative specificity of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) contrasts with the non-specific degeneration of both 5-hydroxytryptamine (5-HT) and dopamine (DA) neurones induced by the indolamine neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) following intracerebral injection (Saner, Pieri, Moran, Da Prada & Pletscher, 1974). Recent studies suggest that ascending 5-HT neurones may modulate the output of nigrostriatal DA mechanisms. When these effects are investigated using the rotating rat model (Ungerstedt & Arbuthnott, 1970), circling responses to both direct and indirect DA agonists are potentiated by drugs attenuating whole brain 5-HT transmission while treatments enhancing 5-HT transmission decrease such rotation (Milson & Pycock, 1976). To investigate this interaction further, circling responses have been investigated following unilateral depletion of DA and unilateral rather than whole brain depletion of 5-HT induced by injection of neurotoxins into the median forebrain bundle (MFB) (Costall, Naylor & Pycock, 1975).

Male Sprague-Dawley rats, 150 ± 20 g, were given unilateral stereotaxic injections of 6-OHDA) 8 µg/4 µl saline), or 5,6-DHT (5 µg/4 µl saline) into the MFB. Groups of rats from each lesion procedure received i.p. injections of either apomorphine (1 mg/kg) or (+)-amphetamine (5-mg/kg) 8 days after surgery; they were then placed in automated rotometer bowls for continuous recording of all rotations. Rats were sacrificed and striata assayed spectrophotofluorimetrically for DA and 5-HT content 14 days after lesion. Rotations were assessed conventionally and after orthogonal polynomial transformation.

6-OHDA lesions produced an 85.5% depletion of striatal DA ($P < 0.0005$) together with a 28.0% depletion of striatal 5-HT ($P < 0.05$); 5,6-DHT lesions produced a 53.8% depletion of 5-HT ($P < 0.01$) together with a 84.9% depletion ($P < 0.001$) of DA. 6-OHDA lesioned animals showed the expected contralateral and ipsilateral rotation to apomorphine and amphetamine respectively. 5,6-DHT lesioned animals showed more rapid onset of contralateral rotation to apomorphine compared with 6-OHDA lesioned animals ($P < 0.02$), indicating that unilateral depletion of striatal 5-HT produces the same facilitation of apomorphine-induced rotation in unilaterally DA lesioned animals as bilateral depletion of 5-HT; rotational responses to amphetamine after 5,6-DHT lesions, however, were attenuated when compared with 6-OHDA lesions ($P < 0.05$), contrary to the enhancement seen after bilateral 5-HT reduction (Milson & Pycock, 1976). This suggests a 5-HT inhibition of nigrostriatal output which, when reduced by unilateral 5-HT lesion enhances apomorphine effects on the ipsilateral side; this procedure attenuates the effects of amphetamine, which acts on the contralateral side, by enhancing the opponent ipsilateral output.

These results suggest that the influence of 5-HT on DA-dependent rotation is exerted at sites within the striatum rather than on the cell bodies or terminals of the nigrostriatal DA pathway. In addition, they emphasize the non-specificity of the degenerative effects of 6-OHDA and 5,6-DHT on catecholamine and indolamine neurones, especially when applied to densely packed monoamine pathways in the MFB.

J.L.W. is an MRC scholar. The transformation analysis was performed by Dr C.D. Frith.

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# Stereochemical specificity in the antipsychotic effects of flupenthixol in man

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Deniker (1960) suggested that ability to induce extrapyramidal side-effects was related to the therapeutic action of chlorpromazine in schizophrenia. Drugs with antipsychotic effects exert actions on central dopaminergic mechanisms (Carlsson & Lundqvist, 1963; O'Keefe, Sharman & Vogt, 1970) and studies of the sensitive adenylate cyclase in the corpus striatum suggest that ability to block the dopamine receptor correlates well with the therapeutic effects of these drugs (Miller, Horn & Iversen, 1974). It has been demonstrated that the two isomers of the thiaxanthene flupenthixol differ widely in their ability to block the dopamine receptor (Miller *et al.*, 1974) although they resemble each other in several other pharmacological properties (Enna, Bennett, Burt, Creese & Snyder, 1976). We have investigated whether these two isomers differ in their therapeutic effects.

Forty-five patients with acute schizophrenic illnesses diagnosed according to Present State Examination criteria were randomly allocated to $\alpha$-flupenthixol, $\beta$-flupenthixol and placebo in a dose of 6 mg for 6 days followed by 9 mg for 22 days. All patients received orphenadrine HCl (50 mg) three times a day, and where additional medication was urgently required chlorpromazine (100 mg). Mental state ratings were made before and at weekly intervals during the trial. All patients showed a decrease in total symptoms over the trial period. This decrease was significantly greater in the group of patients treated with $\alpha$-flupenthixol, than in the groups treated with $\beta$-flupenthixol and placebo. The overall improvement on placebo was slightly greater than on $\beta$-flupenthixol.

These findings demonstrate that the therapeutic potency of flupenthixol is confined to the $\alpha$-isomer and therefore on the basis of *in vitro* receptor studies (Miller *et al.*, 1974; Enna *et al.*, 1976) eliminate the possibilities that the antipsychotic effects are related to blockade of acetylcholine, noradrenaline, GABA, glycine or opiate receptors.

We are grateful to Lundbeck Ltd. for making available preparations of the isomers of flupenthixol.

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## Central dopaminergic and noradrenergic components of bromocryptine-induced locomotor activity in mice

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Bromocryptine (2-bromo-α-ergocryptine; CB 154) has therapeutic activity in Parkinson's disease, attributed to a stimulation of central dopamine (DA) receptors (Corrodi, Fuxe, Hokfelt, Lidbrink & Ungerstedt, 1973; Fuxe, Corrodi, Hokfelt, Lidbrink & Ungerstedt, 1974; Johnson, Loew & Vigouret, 1976). Degeneration of both DA and noradrenaline (NA) neurones may contribute to the symptoms of Parkinsonism. We have therefore examined the roles of both DA and NA in bromocryptine-induced locomotor activity in mice and related these to biochemical changes.

Bromocryptine (10 mg/kg, i.p.) stimulates locomotor activity in normal mice, an effect that is inhibited by pretreatment with pimozide (1 mg/kg, i.p.), FLA 63 (bis 4-methyl-1-homopiperazinylthiocarbonyl disulphide; 25 mg/kg, i.p.) and phenoxybenzamine (20 mg/kg, i.p.) but not by propranolol (20 mg/kg, i.p.). Reserpine (10 mg/kg, i.p. 18–24 h previously) markedly reduced and α-methyl-*p*-tyrosine (AMPT; 200 mg/kg, i.p.) completely abolished, bromocryptine (10 mg/kg, i.p.) induced locomotor activity. This suggests that the locomotion induced by low doses of bromocryptine depends on the functional integrity of synthesis and storage of both DA and NA. The fall of whole brain NA and DA following AMPT (200 mg/kg 1 h prior to death) was reduced 2 h after bromocryptine (5 mg/kg, i.p.) during the period of hyperactivity. In conjunction with the behavioural results, this suggests feedback inhibition of both DA and NA neurones due to receptor stimulation.

High doses of bromocryptine (20–160 mg/kg, i.p.), while causing hyperactivity after a delay, cause a marked initial suppression of locomotion, which can be reversed by administration of apomorphine (2 mg/kg, i.p.) or clonidine (2 mg/kg, i.p.).

Bromocryptine (40 mg/kg, i.p.) enhanced the AMPT fall in NA 2 h following administration during the period of behavioural suppression, but had no effect on DA levels. This suggests that the higher doses of bromocryptine inhibited NA receptors and, indeed, the *in vitro* mouse limbic forebrain NA ($10^{-5}$M) sensitive adenylate cyclase system was inhibited by the addition of bromocryptine ($IC_{50}$ $9.4 \times 10^{-9}$M). The ability of bromocryptine to interact with DA systems was, however, confirmed by the ability of the drug ($10^{-10}$ to $10^{-6}$ M) to displace [$^3$H]-haloperidol for its binding site in rat striatal preparations ($IC_{50}$ $4.3 \times 10^{-8}$ M).

The findings are interpreted as showing a complex involvement of both cerebral NA and DA in the locomotor activity produced by bromocryptine in rodents. The effects of this drug appear to be dose-dependent and may also involve actions on both pre- and post-synaptic monoamine sites.

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## Location of receptors mediating hypothermia after injection of dopamine agonists in rats

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The intracerebroventricular injection of dopamine agonists in rodents causes hypothermia, which is blocked by pimozide (Kruk, 1972). The location of the receptors involved is uncertain, but from indirect evidence striatal (Glick & Marsanico, 1974) or hypothalamic (Cox, Ary & Lomax, 1975) sites have been postulated.

We have attempted to define the site of action more precisely by injecting dopamine and apomorphine into the lateral ventricle, caudate nucleus, third ventricle and preoptic-anterior hypothalamus. Injections were made in a dose volume of 1 µl through previously implanted guide cannulae. Both rectal and tail skin

**Table 1** Effect of intracerebral injection of apomorphine and dopamine

| | | *Mean change in core temperature (°C ± s.e. mean)* | | | |
|---|---|---|---|---|---|
| *Drug* | | *PO/AH* | *CN* | *IIIV* | *LV* |
| Saline | | $+0.2 \pm 0.1$ | $+0.1 \pm 0.1$ | $-0.2 \pm 0.1$ | $+0.4 \pm 0.2$ |
| Apomorphine | 1.25 μg | $-0.3 \pm 0.1^{*}$ | — | $-0.3 \pm 0.2$ | — |
| | 5.0 μg | $-0.8 \pm 0.3^{**}$ | $-0.2 \pm 0.1$ | $-0.3 \pm 0.1$ | $+0.4 \pm 0.1$ |
| | 10.0 μg | $-1.0 \pm 0.2^{**}$ | $-0.2 \pm 0.2$ | $-0.6 \pm 0.1^{*}$ | $+0.3 \pm 0.1$ |
| | 20.0 μg | $-0.9 \pm 0.2^{**}$ | $-0.6 \pm 0.2^{*}$ | $-0.8 \pm 0.3$ | $+0.3 \pm 0.2$ |
| Dopamine | 5.0 μg | $-0.4 \pm 0.1^{*}$ | — | — | — |
| | 10.0 μg | $-0.7 \pm 0.2^{**}$ | — | $-0.5 \pm 0.1$ | $+0.3 \pm 0.1$ |
| | 20.0 μg | $-0.8 \pm 0.2^{**}$ | $-0.2 \pm 0.1$ | — | — |

Mean of maximum change in core temperature occurring within 40 min of injection into preoptic-anterior hypothalamus (PO/AH), caudate nucleus (CN), third ventricle (IIIV) and lateral ventricle (LV).
$n$ = between 4 and 11 observations, $^{*}P < 0.05$, $^{**}P < 0.01$.

temperatures were measured. Although a fall in core temperature was always preceded by a rise in skin temperature, the change in core temperature was more consistent and was used in the expression of results (Table 1). Of the sites tested the preoptic-anterior hypothalamus was the most responsive. When equieffective doses of apomorphine were compared, the time for maximum response was significantly shorter after intrahypothalamic injection (5 μg = 7.0 ± 2.0 min) than after either third ventricular (10 μg = 18.5 ± 1.4 min) or intracaudate injection (20 μg = 20.0 ± 5.7 min).

These results suggest that the most likely site of action of dopamine and apomorphine after intracerebral injection is the preoptic-anterior hypothalamus. That dopamine receptors are involved is indicated by the finding that an i.p. injection of pimozide 0.5 mg/kg antagonized the effect of intrahypothalamic injections of each of the agonists.

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## Comparison of the effects of dopamine and noradrenaline on single cortical neurones

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Cortical neurones can respond with both excitation and depression to microelectrophoretically applied noradrenaline (NA) (Johnson, Roberts, Sobieszek & Straughan, 1969). We have recently presented evidence that the excitatory responses are mediated by $\alpha$-adrenoceptors, whereas the depressant responses are mediated by $\beta$-adrenoceptors (Bevan, Bradshaw & Szabadi, 1976). Cortical neurones are also sensitive to dopamine (DA), both excitatory and depressant responses having been described (Bevan, Bradshaw & Szabadi, 1975; Stone, 1976). However, it has not yet been established whether DA and NA act at pharmacologically distinct receptors on these cells.

Single spontaneously active neurones were studied in the somatosensory cortices of halothane-anaesthetized rats. All the drugs were applied by microelectrophoresis.

The direction of the responses (excitation or depression) evoked by DA and NA were compared on 46 cells. All these cells responded in the same direction to the two catecholamines, 30 being excited and 16 being depressed by both drugs.

We have compared the effects of the α-adrenoceptor antagonists phentolamine and phenoxybenzamine and the neuroleptic drug haloperidol (a drug which is believed to block DA receptors, see Woodruff, 1971) on excitatory responses to DA and NA. Acetylcholine (ACh) was used as a control agonist. The effects of phentolamine were studied on 4 cells. On all of these cells phentolamine reversibly antagonized excitatory responses to NA and DA without affecting excitatory responses to ACh. On one of the cells the excitatory responses to both NA and DA were reversed into depressant responses in the presence of phentolamine.

The effects of phenoxybenzamine were examined on 8 cells. On all the cells tested phenoxybenzamine reversibly antagonized the response to NA without affecting the response to ACh. On 5 cells the response to DA was also antagonized; however, on the remaining 3, phenoxybenzamine partially discriminated between the responses to NA and DA in that the response to DA was affected to a much lesser degree than was the response to NA.

The effects of haloperidol were tested on 6 cells. On 5 cells, the response to DA was antagonized when the response to ACh was not affected. On 4 of these, the response to NA was affected to a lesser degree than was the response to DA.

The partial selectivity of phenoxybenzamine and haloperidol may indicate that the excitatory responses to the catecholamines are mediated by two populations of receptors. One population (probably α-adrenoceptors; Bevan *et al.*, 1976) may be blocked by phenoxybenzamine, and may be more sensitive to NA than to DA. The other population may be blocked by haloperidol, and may be more sensitive to DA than to NA. It remains to be determined whether there are pharmacologically distinct receptors mediating the depressant effects of DA and NA on cortical neurones.

This work was supported by the North Western Regional Health Authority and the Mental Health Trust and Research Fund.

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## On the depressant action of dopamine in rat caudate nucleus and nucleus accumbens

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The putative neurotransmitter dopamine has both inhibitory and excitatory actions on neurones. In our own studies we have found that iontophoretically-applied dopamine causes mainly depression of firing of cells in the rat caudate nucleus and nucleus accumbens; however, several workers have reported excitatory actions of dopamine in the caudate nucleus (for references see Woodruff, McCarthy & Walker, 1976).

Recently, Kitai, Sugimoro & Kocsis (1976) suggested that the depressant action of dopamine in the striatum might be due to an excitation of inhibitory interneurones. If this were so, the depressant action of dopamine should be prevented by antagonists of the inhibitory transmitter involved.

Extracts of rat striatum or nucleus accumbens contain appreciable amounts of γ-aminobutyric acid (GABA), glutamate, glycine, aspartate and taurine. In the present study we have examined the possibility that one of the above amino acids might be involved in the inhibitory actions of dopamine.

Extracellular recordings were made from neurones and drugs were applied microiontophoretically, using techniques described by Crossman, Walker & Woodruff (1974). Neurones were either spontaneously active or were driven by DL-homocysteic acid (100 mM, pH 9.0). Other drugs were applied from 100 mM solutions at pH 3.5, except strychnine (6 mM, pH 3.5), picrotoxin (saturated solution in 150 mM NaCl), glutamate and aspartate (each 100 mM, pH 9.0) and $\alpha$-flupenthixol (200 mM, pH 2.0).

The activity of neurones in both the nucleus accumbens and caudate nucleus was consistently depressed by GABA (10–30 nA), glycine (10–30 nA), dopamine (10–50 nA) or taurine (10–50 nA). Glutamate and aspartate caused excitation of all cells tested. Strychnine (60 nA for 4 min) reversibly blocked the depression produced by glycine (24 neurones), but had no effect on responses to GABA or dopamine in either the nucleus accumbens (12 cells) or caudate nucleus (8 cells). In both regions of the brain picrotoxin (70 nA for 5 min) reversibly antagonized the inhibitory actions of GABA and taurine, but had no effect on responses to glycine (14 cells) or dopamine (16 cells). $\alpha$-Flupenthixol selectively blocked the depressant action of dopamine on 9 out of 20 neurones in the caudate nucleus and on 12 out of 22 cells tested in the nucleus accumbens. $\alpha$-Flupenthixol has been shown to have a similar action in cat putamen and amygdala (Ben-Ari & Kelly, 1976).

Our results suggest that, although GABA, taurine and glycine might be inhibitory transmitters in the nucleus accumbens and caudate nucleus, it is unlikely that they are involved in mediating the depressant actions of dopamine in these regions of the brain.

We are grateful to the M.R.C. for an apparatus grant, and for a training grant to P.S.M. The flupenthixol was generously donated by Dr Møller Nielsen of Lundbeck.

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# The effect of iron deficiency on brain monoamine metabolism and the behavioural responses to increased brain 5-hydroxytryptamine and dopamine synthesis

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Iron has been suggested to be a co-factor for monoamine oxidase (see Youdim, 1976), tryptophan and tyrosine hydroxylase. It has been shown that iron deficiency anaemia lowers the activity of monoamine oxidase both in rat liver (Symes, Sourkes, Youdim, Gregoriadis & Birnbaum, 1969; Symes, Missala & Sourkes, 1971) and human platelets (Youdim, Woods, Mitchell, Grahame-Smith & Callender, 1975). In the brain these enzymes are involved in the synthesis and catabolism of the monoamine neurotransmitters. However there has been little investigation of the effects of iron deficiency anaemia on the metabolism and function of 5-hydroxytryptamine (5-HT), dopamine (DA) or noradrenaline (NA). We have now investigated the effect of iron deficiency anaemia in rats on various enzymes involved in central monoamine metabolism and on the functional activity of 5-HT and DA.

Rats were fed a semi-synthetic diet lacking in iron (McCall, Newman, O'Brien, Valberg & Witts, 1962) and distilled water. Control rats were given the same diet with iron added and tap water. After approximately 5 weeks when the rats were iron-deficient (haemoglobin (g/dl); control $14.2 \pm 0.44$ (16) experimental: $5.55 \pm 0.42$ (22), $P < 0.001$) it was found that brain non-haem iron stores were decreased by 60%. At this time the activities of tryptophan hydroxylase, aldehyde dehydrogenase and monoamine oxidase were unaltered in the brain.

The concentration of brain 5-HT was somewhat decreased (control $0.38 \pm 0.02$ µg 5-HT/g brain (wet wt) (6); experimental $0.31 \pm 0.02$ (9), $P < 0.01$) possibly because of decreased 5-HT binding at the nerve ending, since $Fe^{++}$ has been implicated in this process (Tamir, Klein & Rapport, 1976). However, DA and NA concentrations were unaltered, as was the rate of 5-HT synthesis.

The iron-deficient rats showed inhibition of the hyperactivity response following administration of tranylcypromine (20 mg/kg) and L-tryptophan (100 mg/kg) a procedure which increases brain 5-HT synthesis, despite the rate of 5-HT accumulation being the same in both groups. (Total movements in the 90 min following L-tryptophan, control 8680 ± 1102, experimental 3468 ± 166, 3 observations each, $P < 0.01$). The hyperactivity response to the suggested 5-HT agonist, 5-methoxy *N,N*-dimethyl tryptamine was also inhibited in these animals indicating an interference with the mechanisms by which 5-HT brings about the hyperactivity syndrome (see Green & Grahame-Smith, 1976).

The locomotor response of iron-deficient rats to tranylcypromine (20 mg/kg) and L-dopa (100 mg/kg) was also inhibited although there was no difference in the accumulation of DA and NA from controls.

The response to methamphetamine (2 mg/kg) and apomorphine (2 mg/kg) was also decreased. This suggests that DA responses were inhibited in the iron deficient-group.

The DA and 5-HT mediated responses became normal after feeding the iron-deficient group with the iron-plus diet for 7 days. At this time the haemoglobin values approached normal (12.2 ± 0.42 g/dl (6)).

These results will be discussed in relation to the role of iron in the mechanisms of monoamine neurotransmission.

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## Ultrastructural changes in the coronary vascular system following prolonged emotional stress. An experimental model for the study of coronary vascular disease?

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Following exposure of the rat to a variety of stressful situations there occurs an elevation of circulating corticosterone (11-OHCS). Work from this laboratory indicates that the elevation can be quantitated, in that rats exposed to signalled regular footshock (2 s of 2 mA scrambled footshock every 88 s for 45 min, i.e. 30 trials) show an intermediate (45–55 µg/100 ml blood plasma), and that rats exposed to irregular signalled footshock with the possibility of escape (7 shock exposures randomly placed in a 35 min session) show extreme (85–95 µg/100 ml blood plasma) (Bassett, Cairncross & King, 1973) steroid elevation. Intermediate 11-OHCS elevation is independent of the physical intensity of the stressor, and the extreme 11-OHCS elevation occurs only with unpredictability of stress. The contention is made therefore, that extreme steroid elevation contains a psychological component which when superimposed on the physical stress response produces an increment in 11-OHCS output (Cairncross & Bassett, 1975).

The 'psychological increment' of the stress response appears to be linked with unpredictability in the timing of the stressor, and might be considered of emotional derivation. Such a derivation has long been implicated in the pathogenesis of cardiovascular disease. It seemed not unreasonable therefore to expose male rats to the irregular signalled footshock regimen over a prolonged period of time (70 days) and to examine during and at the end of that period, not only the degree of 11-OHCS elevation, but also the morphological changes which could predictably occur within the cardiovascular system and within other physiological systems.

The morphological changes obtained with light microscopy were largely confined to the microcirculation of the coronary vasculature, and included, at 70 days, a significant degree of congestion and dilation of the large venules, collecting venules and veins. There was accumulation of PAS staining positive material, consistent with platelet aggregation marginated on the endothelium of small veins. Coronary arterioles showed some oedema of the intima and media with the presence of microvacuoles. There was a marked mast cell infiltration into perivascular areas of the coronary microcirculation.

In view of the results described it was necessary (i) to confirm that the microvacuoles contained lipid, (ii) to examine the ultrastructure of the endothelial lining for evidence of junctional gaps, and (iii) to search for indications of platelet aggregation within the microcirculation. Evidence is presented confirming each of the suppositions enumerated.

A time course study designed to demonstrate the onset of ultrastructural changes indicates that junctional gaps appear after 40 days of stress. Examination of plasma 11-OHCS levels indicates that the extreme elevation is maintained for at least 25 days, and thereafter declines to some 45 µg/100 ml plasma by day forty. This level of 11-OHCS is maintained through to termination of the experiment at day seventy. It is possible that a causal relationship exists between the microcirculatory changes and the reduction in circulating 11-OHCS levels.

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## The effect of some anti-inflammatory and anti-malarial drugs on the migration of horse leucocytes *in vitro*

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When leucocytes are packed into a capillary tube, by centrifugation, and the cell-supernatant interface is cut, they migrate fanwise from the cut end. The process has been studied extensively in relation to delayed hypersensitivity, and recently as affected by some anti-inflammatory drugs (Meacock & Kitchen, 1976). We also have examined the effects of some anti-inflammatory drugs, particularly those which also have anti-malarial activity. Migration has been inhibited by a number of such drugs, and also by chemically related compounds not known to be anti-malarial or anti-inflammatory.

Leucocytes have been obtained by allowing erythrocytes to sediment spontaneously from horse blood, yielding a leucocyte-rich plasma, which is centrifuged at 180 *g* for 10 minutes. The leucocytes are resuspended in medium 199 and are allowed to migrate for 18 h as described by George & Vaughan (1962), with minor modifications, in the presence of drug. The leucocytes comprise 80.0% (s.e. mean $= \pm 1.8$, $n = 14$) polymorphonuclear neutrophils. Migration is measured as the distance from the cut end of the capillary to the boundary of the migrating cells. Conventionally, in experiments of this type the response is measured as the area covered by the migrating cells. However, we have found that the variance of such measurements was strongly correlated with the mean ($r = +0.53$, $n = 20$, $P = 0.01$) whereas the same parameters of the distance migrated was only slightly correlated ($r = 0.21$, $n = 20$, $P = 0.34$). Potency of compounds has therefore been estimated as the concentration of drug reducing the distance of migration to 50% of the control.

The following anti-malarial drugs were tested by this method: mepacrine, chloroquine, hydroxychloroquine, chloroguanide, cycloguanil, primaquine and quinine. Drugs of similar chemical structure such as imipramine and chlorpromazine, together with aspirin, indomethacin, phenyl butazone and hydrocortisone were also examined.

We have found that mepacrine, imipramine, chlorpromazine and proguanil all inhibited leucocyte migration with $EC_{50}$ of between 90 and 250 µM. Chloroquine, hydroxychloroquine and quinine were about 8 times less active than mepacrine and primaquine 5 times less so. Cycloguanil and conventional anti-inflammatory drugs were inactive up to 1000 µM.

These findings confirm that conventional anti-inflammatory drugs do not inhibit random migration of polymorphonuclear neutrophils. Mepacrine and chloroquine, which have been used to control rheumatoid arthritis, are active at concentrations comparable to those achieved therapeutically. It appears that inhibition of migration is neither specific to anti-malarial activity nor to chemical structure, but this activity may contribute to the anti-inflammatory activity of these drugs.

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## On the ineffectiveness of indomethacin against rheumatoid swelling

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Previously, it was suggested that indomethacin-like drugs fail to arrest rheumatoid swelling because of their inability to prevent the inflammatory changes initiated by circulating sensitized lymphocytes (Jasani, 1975). The effectiveness of indomethacin against lymphocyte-initiated swelling and the accumulation of fibrin has been tested using the skin graft model (Bach & Jasani, 1976) and compared with that of two cortisol-like steroids: fluocinolone acetonide and chlorflumethasone.

To secure the development of circulating sensitized lymphocytes in the test animal, six homografts were transplanted on to each hind limb of the recipient rabbit and the drug was applied topically to one set only (Jasani, Parsons, Roberts & Tweed, 1974). The placebo-treated contralateral set provided the antigenic stimulus leading to the emergence of circulating sensitized lymphocytes (Jasani, 1976).

The results showed that indomethacin (0.02%) was virtually ineffective against the swelling in homografts; a failure which cannot be due to lack of absorption of topically applied indomethacin, which is known to be as active topically as when administered orally (Lewis, 1976). In contrast, the two steroids were definitely effective against both the inflammatory accompaniments. Compared with indomethacin which reduced the swelling only slightly (96.6 ± 0.9%) and tended to increase fibrin accumulation (110.6 ± 4.7%), fluocinolone acetonide (0.0025%) reduced the swelling and fibrin content to 66.2 ± 6.3% and 53.6 ± 6.0% respectively of control, whereas chlorflumethasone (0.00025%) reduced them to 62.2 ± 5.1% and 69.2 ± 1.9% (mean ± s.e. mean, $n = 4$).

As the accumulation of fibrin occurs in the rheumatoid joint in a manner similar to that in homografts (Bullock, Jasani & Roberts, 1976), the observations suggest that new anti-rheumatic agents should also exhibit steroid-like properties in their ability to depress lymphocyte-initiated inflammation but should be devoid of undesirable effects.

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## Site of action of the antiallergic drugs cromoglycate and doxantrazole

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The degree of antigen-induced histamine secretion from sensitized mast cells is correlated with 45-calcium uptake (Foreman, Hallett & Mongar, 1975). Inhibition of oxidative and glycolytic metabolism prevents histamine secretion but not $^{45}$Ca uptake, whereas dibutyryl cyclic AMP inhibits both $^{45}$Ca uptake and secretion. Histamine secretion induced by the calcium ionophore, A23187 is not inhibited by dibutyryl cyclic AMP or by antiallergic drugs (Foreman, Mongar, Gomperts & Garland, 1975) indicating that these inhibitors exert their effect on antigen-induced secretion at the level of calcium entry into the mast cell.

The methods of measuring histamine secretion from, and $^{45}$Ca uptake by sensitized rat peritoneal mast cells have already been described (Foreman, Mongar & Gomperts, 1973).

Cromoglycate and doxantrazole produce a dose-related inhibition of antigen-stimulated $^{45}$Ca uptake by the mast cells. The concentration ranges for inhibition of $^{45}$Ca uptake are similar to those for inhibition of histamine secretion (Garland & Mongar, 1976). Inhibition of $^{45}$Ca uptake by dibutyryl cyclic AMP is

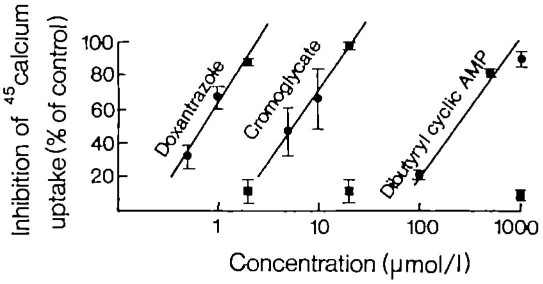

**Figure 1** Inhibition by doxantrazole, cromoglycate and dibutyryl cyclic AMP of $^{45}$Ca uptake induced by antigen (●) or A23187 (■) from sensitized rat peritoneal mast cells. Inhibition is expressed as a percentage of $^{45}$Ca uptake in the absence of inhibitor which was 462 ± 158 ct/min for antigen and 2637 ± 459 ct/min for A23187. $^{45}$Ca uptake in the absence of stimulus was 76 ± 14.6 ct/min. Uptakes refer to counts associated with approx. $5 \times 10^5$ cells and means are given together with s.e. mean for three experiments. The histamine secretions in the absence of inhibitor were 26 ± 2.8% for antigen and 55% ± 3.1 for A23187. Concentration of antigen was supramaximal and that of A23187 was 3.0 μmol/l.

compared with that produced by the antiallergic drugs in Figure 1. Relative to cromoglycate, doxantrazole is eight times more effective in inhibiting $^{45}$Ca uptake, whereas dibutyryl cyclic AMP is only 0.025 times as active. The activities for the inhibition of histamine secretion, cromoglycate: doxantrazole: dibutyryl cyclic AMP: are 1 : 20 : 0.02. None of the three agents inhibits $^{45}$Ca uptake (Figure 1) or histamine secretion induced by the calcium ionophore, A23187.

Experiments with the ionophore A23187 have suggested that calcium entry into the mast cell is a sufficient stimulus to secretion (Foreman *et al.*, 1973). The antigen-antibody reaction appears to allow entry of calcium into the cell by increasing membrane permeability. The results presented here show that cromoglycate and doxantrazole inhibit the antigen-induced $^{45}$Ca uptake and may exert their antisecretory effects by this mechanism. It is unlikely that these agents act at a point after calcium entry into the cell since they do not inhibit ionophore-induced secretion. The antiallergic agents are also inhibitors of phosphodiesterase (Roy & Warren, 1974; Tateson & Trist, 1976) and may prevent $^{45}$Ca uptake indirectly by raising intracellular levels of cyclic AMP, since dibutyryl cyclic AMP itself prevents calcium uptake.

M.B.H. is an MRC scholar.

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## Dependence of histamine release from rat mast cells induced by the ionophore A23187 on endogenous adenosine triphosphate

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Pure populations of rat peritoneal mast cells were used to study the ATP content of the mast cell in relation to histamine release induced by the ionophore A23187. When the ATP content was reduced to varying levels by preincubation with antimycin A and A23187, a good correlation was found between the ATP levels of the mast cells and the amounts of histamine released by incubation with calcium. The time course of A23187-induced histamine release and the effect of A23187 on the ATP content of the mast cells were studied under aerobic and anaerobic conditions. Histamine release was completed within 10 min from cells incubated under aerobic conditions, and there was a reduction in the ATP content of the cells incubated in the presence or absence of glucose in close time relation to the histamine release. In the anaerobic experiments antimycin A was used to block the oxidative phosphorylation. After preincubation with antimycin A, glucose and A23187, calcium was added to the cell suspension. There was a marked reduction in the ATP content during the same period, when histamine release occurred. In other anaerobic experiments oligomycin was added to the cells during the preincubation period together with antimycin A, glucose and A23187. By incubation with calcium the ATP content of the mast cells was reduced during the histamine release. The observations are consistent with the view that energy requiring processes are involved in A23187 induced histamine release from mast cells, although part of ATP reduction in the aerobic experiments may be due to an uncoupling effect of $Ca^{++}$ on the oxidative phosphorylation.

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# The effect of histamine antagonists on antigen-induced contractions of sensitized human bronchus *in vitro*

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The nature and mechanisms of the participation of histamine in bronchial asthma are unclear despite its long history. The object of this work was to identify the relative importance of histamine in allergic bronchospasm in a passively sensitized human bronchial muscle preparation.

Human bronchus, 2–3 mm in diameter, dissected free of macroscopically normal tissue removed for carcinoma, was passively sensitized in undiluted serum from patients with house dust mite allergy. Sensitized bronchi were cut into spiral strips and suspended in 10 ml organ baths under a load of 250 mg, perfused with Krebs at 37°C and gassed with 95% $O_2$ and 5% $CO_2$. Changes in muscle length were recorded electronically with an isotonic transducer and recorded on moving paper. When the muscle tone was steady responses to a maximum dose of acetylcholine chloride (200 µg/ml for 3 min) and to a series of doses of histamine acid phosphate (5, 20, 80 µg/ml for 5 min) were obtained for each preparation. This was repeated after half the tissues had been treated with the inhibitor being tested, the remainder being parallel controls. Finally, all the tissues were challenged with freeze-dried house dust mite antigen (D. Pteronyssius 10 µg/ml). These responses were expressed as a percentage of the maximum acetylcholine response (MAR) recorded prior to challenge to enable comparison between tissues. In two series of experiments the effect of the $H_1$ receptor antagonist mepyramine (25 µg/ml), and the $H_2$ receptor antagonist metiamide (10 µg/ml) were studied on the dose response curve to histamine and response to antigen challenge. The effect of metiamide on a submaximal challenge (0.1 µg/ml D. Pteronyssius antigen) was also studied.

Dose related bronchial muscle contraction occurred in response to histamine but in the presence of mepyramine dose related relaxation occurred which was subsequently abolished by metiamide.

Antigen challenge caused contraction of the muscle in all cases. Mepyramine significantly reduced this response from $117.3 \pm 6.2$ in controls to $89.2 \pm 8.3\%$ MAR ($P < 0.005$, $n = 53$). Metiamide alone increased the response to histamine when compared with parallel controls, but it has no significant effect on the maximum antigen challenge response which was $124.0 \pm 10.0$ in controls and $114.6 \pm 10.3\%$ MAR in metiamide treated tissues ($P > 0.05$, $n = 21$). It did, however, cause a significant increase in the submaximal antigen challenge response from $81.2 \pm 7.1$ in controls to $116.4 \pm 21.1\%$ MAR ($P < 0.02$, $n = 42$). The challenge response in the presence of the two antagonists together, $109.3 \pm 5.1\%$ MAR, was not significantly different from the parallel control experiments, $107.9 \pm 6.2\%$ MAR ($P > 0.05$, $n = 38$).

The results of the experiments indicate that both $H_1$ and $H_2$ receptors occur in human bronchus and that both participate in allergic bronchospasm, $H_1$ receptors stimulating and $H_2$ receptors inhibiting muscle tone. Their effects, however, appear to cancel each other out, which suggests that if histamine plays any role in asthmatic bronchospasm, it is more likely to be via an indirect (DeKock, Nadel, Zwi, Colebatch & Olsen, 1966) rather than a direct action.

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## Are gastrin receptors located on parietal cells?

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Gastric acid secretion stimulated by either food or pentagastrin is as sensitive to inhibition by histamine $H_2$-receptor antagonists as is histamine-stimulated secretion. This could be explained if parietal cells have separate receptors for histamine and gastrin such that blockade of one alters the other (Grossman & Konturek, 1974). Alternatively, histamine may act directly on parietal cells and gastrin indirectly by releasing histamine from local histamine-secreting cells (Kahlson & Rosengren, 1972). Secretion of histamine from rat mast cells is critically dependent on calcium (Foreman & Mongar, 1972), while stimulation of cat salivary secretion, particularly of electrolytes, is not (Martinez & Petersen, 1972). If gastrin releases histamine, then there is a possibility that the two exogenous agonists exhibit different degrees of calcium dependence.

This possibility was tested on isolated, lumen perfused, mouse stomach preparations, suspended in organ baths at 37°C (Wan, 1975). The calcium concentration of the unbuffered lumen perfusate was 1.3 mM and that of the phosphate buffered bathing solution was 0.65 mM. Agonists were added to the bath, but to avoid desensitisation, each stomach was only equilibrated with one agonist concentration. The pH of the lumen-perfusate was sensed by a flow electrode system. The peak secretory responses were calculated by the change in hydrogen ion concentration and expressed as $\Delta H^+$ nmoles/ml. Submaximal doses of histamine $10^{-5}$M and pentagastrin $10^{-7}$M were given in a randomized block design with six preparations run concurrently.

The mean and standard error of the response to histamine ($n=6$) was $202 \pm 34$ and that to pentagastrin $101 \pm 31$. In the absence of calcium, the responses were $141 \pm 32$ ($P > 0.1$) and $15 \pm 5$ ($P < 0.05$) respectively.

Since magnesium can reduce the release of histamine through competitive inhibition of $Ca^{++}$ uptake (Foreman & Mongar, 1972), mouse stomachs were exposed to the bathing solution and perfusate containing 5 or 20 mM $Mg^{++}$ replacing sodium. Histamine responses in control and 5 mM $Mg^{++}$ solutions were $135 \pm 11$ and $114 \pm 22$ ($n=6$, $P > 0.2$) respectively; corresponding pentagastrin responses were $158 \pm 19$ and $46 \pm 16$ showing significant inhibition ($n=6$, $P < 0.001$). No further inhibition of pentagastrin responses occurred at 20 mM $Mg^{++}$, but histamine responses were significantly increased to $303 \pm 31$ ($n=6$, $P < 0.05$).

The results could be interpreted so that histamine and pentagastrin still act on the same cell if histamine mobilised intracellular $Ca^{++}$ and pentagastrin increased the membrane permeability to $Ca^{++}$. However, our finding that acid secretion evoked by the calcium ionophore, A23187 (Foreman, Gomperts & Mongar, 1973), can be inhibited by metiamide, is incompatible with this hypothesis unless metiamide is also a calcium antagonist. Since there is no evidence for this, our results therefore reinforce the hypothesis that gastrin stimulates acid secretion indirectly, via histamine secreting cells.

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# Interaction between histamine $H_2$-receptor antagonists and the hypotensive effects of clonidine in rats

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Clonidine is a potent centrally acting antihypertensive agent, the major action of which has been considered to be due to central $\alpha$-adrenoceptor stimulation (Schmitt, Schmitt & Fenard, 1973). Recently, however, hypotension elicited by peripheral administration of clonidine to rats has been reported to be antagonized by central administration of the histamine $H_2$-receptor antagonist, metiamide, (Karppannen, Paakkari, Huotari & Orma, 1976; Paakkari, Paakkari & Karppannen, 1976) thus suggesting a possible central involvement of histamine receptors with the hypotensive effects of clonidine. Furthermore, clonidine appears to stimulate histamine $H_2$-receptors in the heart *in vitro* (Csongrady & Kobinger, 1974) and to stimulate cAMP accumulation in rat brain slices by interaction with histamine $H_2$-receptors (Audigier, Virion & Schwartz, 1976). In conscious cats however, metiamide failed to antagonize hypotension after central administration of clonidine (Finch & Hicks, 1976). The present study reports further findings on the interaction between clonidine and the histamine $H_2$-receptor antagonists metiamide and cimetidine.

Rats were anaesthetized with urethane (1.25 g/kg i.p.). The trachea was cannulated and arterial blood pressure measured. Drugs were injected into lateral brain ventricles (i.c.v.) or via an intravenous catheter.

Intravenous administration of clonidine (30 μg/kg) caused a fall in mean blood pressure. In one group, clonidine lowered blood pressure by $46.3 \pm 6.9$ mm Hg (mean ± s.e. mean, $n = 8$) in rats pretreated with saline i.c.v. but only $14.8 \pm 4.5$ mm Hg in rats pretreated with metamide (400 μg i.c.v.); metiamide (800 μg) abolished the responses to clonidine. Metiamide reduced but did not abolish the bradycardia due to clonidine. Cimetidine, although less effective than metiamide, also antagonized the hypotensive response to clonidine.

Treatment with either $H_2$-receptor antagonist i.c.v. 15–30 min after clonidine (30 μg/kg) produced a rapid reversal of the hypotensive response to clonidine although this was not well sustained. The smallest effective dose of metiamide was 50 μg and total reversal was achieved by all doses of either antagonist in excess of 100 μg ($n = 6$ per group). In contrast, metiamide (400 μg) given intravenously did not reverse clonidine hypotension.

Administration of either metiamide or cimetidine, (400 and 800 μg i.c.v.) alone caused dose-dependent increases in blood pressure with variable effects on heart rate. Although the antagonists alone increase blood pressure when given in large doses i.c.v. this is unlikely to account for their ability to prevent clonidine-induced hypotension when administered as pretreatments or to reverse the response when administered in lower doses after clonidine.

These results confirm an interaction between clonidine and centrally administered histamine $H_2$-receptor antagonists. However, centrally mediated hypotensive responses to histamine have yet to be demonstrated (Finch & Hicks, 1976) suggesting that it may be premature to implicate histamine $H_2$-receptors in the hypotensive response to clonidine. An interaction between clonidine and cimetidine is not of clinical significance, since autoradiography has indicated that cimetidine does not penetrate the blood brain barrier (Cross, 1976) and no interaction has been observed between clonidine and the histamine $H_2$-receptor antagonists when these are given systematically.

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## Inhibition of $[^3H]$-naloxone binding by opiate agonists

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It has been shown that the relative receptor affinities of most opiates, as measured by their inhibition of $[^3H]$-naloxone binding to rat brain homogenates, correlate well with their pharmacological actions in the guinea-pig ileum, which is a good predictor of their analgesic action in man (Kosterlitz & Waterfield, 1975; Creese & Snyder, 1975). Of particular interest is the fact that $Na^+$ decreases the binding of agonists whilst not affecting, or slightly enhancing, that of antagonists (Pert & Synder, 1974). The purpose of this communication is to draw attention to the great variability of the sodium shift.

A modification of the method of Pert & Snyder was used. Guinea-pig brain homogenates, prepared by means of an Ultraturrax, were spun at 600 *g* to remove cell debris. The supernatant was centrifuged at 49,000 *g* and the resulting pellets resuspended in either 50 mM Tris buffer (pH 7.4 at 37°C) or in Krebs-Tris. Aliquots of homogenate (2 ml) were incubated with 1 nM $[^3H]$-naloxone (4 Ci/mmol, NIDA) plus the inhibiting drug at 37°C for 20 min, the samples then being cooled on ice for 30 min before filtration through Whatman GF/B filters. Stereospecific naloxone binding was determined by subtracting the binding in the presence of 50 nM MR 2266 {(−)-α-5,9-diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan} from that in its absence. For each drug, the concentration required to produce a 50% inhibition of binding was calculated from log-probit regression lines.

The degree of sodium shift was not constant for all agonists since a 7 to 150 fold decrease in potency was observed. Pert & Snyder (1974) found that agonists with an antagonist component have a small sodium shift but the great variation found in this study cannot be explained on this basis, as only pure agonists were used. It is of interest that the three drugs with the smallest sodium shifts belong to a group of benzomorphans which are unusual in that they do not cause morphine-like dependence in monkeys and require more naloxone than does normorphine to antagonize their actions in the guinea-pig ileum and mouse vas deferens (Hutchinson, Kosterlitz, Leslie, Waterfield & Terenius, 1975). They are (−)-α-(1R, 5R, 9R)-5,9-dimethyl-2-(L-tetrahydrofurfuryl)-2′-hydroxy-6,7-benzomorphan (MR 2034, C.H. Boehringer Sohn), (±)-α-5,9-dimethyl-2-(3-methyl-furfuryl)-2′-hydroxy-6,7-benzomorphan (MR 1353) and (±)-9-methyl-5-ethyl-8-oxo-2-cyclopropyl-methyl-2′-hydroxy-6,7-benzomorphan (ethylketocyclazocine, Win 35,197-2, Sterling-Winthrop).

For most of the agonists studied there is good correlation between the relative potencies of inhibition of binding and those found in the pharmacological models, the guinea-pig ileum and mouse vas deferens. However, some compounds, notably Win 35,197-2, were less potent in the binding assay than in the pharmacological models. The reason for this discrepancy is, as yet, unclear. It would, however, appear to be advisable to employ several parallel *in vitro* assays, using pharmacological responses and inhibition of binding, for the prediction of potencies *in vivo*.

Supported by a grant from the National Institute on Drug Abuse (DA-00662). F.M.L. is a MRC Scholar.

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## Dopamine utilization in the posterior pituitary gland of the rat

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Dopamine is present in the posterior lobe of the pituitary gland where it is thought to be contained in nerve fibres (Björklund, 1968; Saavedra, Palkovits, Kizer, Brownstein & Zivin, 1975). As a first step in the study of the function of dopamine (DA) in this tissue, its utilization was investigated. Albino rats (Wistar, Porton strain) were injected with α-methyl-*p*-tyrosine (AMPT), an inhibitor of tyrosine hydroxylase and were killed 30, 60 and 120 min later. Control rats were injected with a 0.9% NaCl solution and killed after the same time intervals. The pituitary gland was dissected out, the anterior lobe separated from the intermediate and posterior lobe and the latter tissues analysed together for their DA content since Saavedra *et al.* (1975) have shown that the posterior lobe contains the majority of the dopamine in the pituitary gland. The tissues from 3 rats were pooled for each sample. The DA content was determined by a radio enzymatic assay (Fry, House & Sharman, 1974). In untreated female rats (200–300 g body weight) 2.12 ± 0.14 ng DA/posterior lobe ($n = 5$ samples) were found, in untreated male rats (250–300 g body weight) the value was 2.19 ± 0.10 ng ($n = 6$ samples). The quantity of DA contained in the anterior lobe was about 0.2 ng/gland. In Table 1 some results on the effect of AMPT on pituitary DA are listed. In a group of female rats (mean body weight 200 g) there occurred a significant reduction in pituitary DA by 28% 30 min after AMPT; after 60 min it was reduced by 63% but no further decrease occurred after 120 minutes. In male rats (mean body weight 320 g) the reduction in pituitary DA 30 min and 60 min after AMPT was not statistically significant but after 120 min the DA content was significantly decreased by 44%. In the same rats a similar rate of fall in the DA concentration of the striatum was observed.

These experiments demonstrate that the DA in the posterior pituitary gland is being utilized. Experiments to study the concentration of pituitary DA under conditions which result in changes in the secretion of pituitary hormones are in progress.

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**Table 1** Effect of $\alpha$-methyl-$p$-tyrosine (AMPT) on the dopamine content of the posterior pituitary and the striatum in the rat

| | | *Dopamine content* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Posterior pituitary (ng/posterior lobe)* | | | *Striatum (μg/g tissue)* | | |
| *Rats age* | *Time after injection (min)* | *Control** | *AMPT*** | *Difference between control AMPT* | *Control** | *AMPT*** | *Difference between control and AMPT* |
| Female 9 weeks | 30 | 1.8 ± 0.07 (2) | 1.30 ± 0.08 (3) | −28%, $P < 0.02$ | — | — | — |
| | 60 | 2.01 ± 0.40 (3) | 0.75 ± 0.13 (3) | −63% $P < 0.05$ | | | |
| | 120 | 1.88 ± 0.15 (3) | 0.81 ± 0.12 (3) | −57%, $P < 0.01$ | | | |
| Male 16 weeks | 30 | 3.07 ± 0.61 (5) | 2.00 ± 0.29 (5) | −35% n.s. | 9.07 ± 0.59 (10) | 7.86 ± 0.60 (9) | −13%, n.s. |
| | 60 | 2.50 ± 0.34 (5) | 1.84 ± 0.42 (5) | −26%, n.s. | 10.04 ± 0.51 (10) | 7.11 ± 0.33 (10) | −29%, $P < 0.01$ |
| | 120 | 2.55 ± 0.09 (5) | 1.44 ± 0.33 (5) | −44%, $P < 0.01$ | 9.16 ± 0.48 (10) | 5.88 ± 0.52 (9) | −36%, $P < 0.01$ |

*: 10 ml 0.9% sodium chloride i.p./kg body weight; **: 200 mg/kg body weight i.p.; number in brackets: number of samples analysed. Posterior lobe: each sample consisted of three posterior lobes. Striatum: each sample contained the left and right striatum of one rat. Statistical analysis by Student's t-test; n.s.: not significant ($P > 0.05$).

# Vasopressin release by antagonists of GABA and glycine

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In cats anaesthetized with chloralose, nicotine releases vasopressin without oxytocin when acting from the ventral surface of the brain stem. To obtain this effect nicotine was injected into the cerebral ventricles or, more usually, applied topically through perspex rings (20 µl. into each ring of a solution of 2 or 4 mg/ml) to a bilateral region lateral to the pyramids and 6 to 9 mm caudal to the trapezoid bodies (Bisset, Feldberg, Guertzenstein & Rocha e Silva Jr, 1975). The vasopressin release occurred almost instantaneously and was short-lasting. It was followed by a paralyzing action rendering the region insensitive to renewed nicotine application. Hexamethonium similarly applied shared with nicotine the paralyzing action but did not itself stimulate vasopressin release (Bisset & Feldberg, unpublished experiments).

Using the procedure of Bisset *et al.*, there was no vasopressin release on topical application of physostigmine (10 to 50 mg/ml) carbachol (6 mg/ml), tetra-ethyl and tetra-methyl ammonium iodide (5–20 mg/ml) to the nicotine sensitive region, nor on injection into the cerebral ventricles of noradrenaline (100 µg) or morphine sulphate (750 µg), the morphine being injected into unanaesthetized cats. But the topical application of tubocurarine (20 mg/ml), for only a few minutes, released large amounts of vasopressin. In contrast to the effect of nicotine the release proceeded gradually and reached its maximum after about 1 hour.

On account of its neuromuscular and ganglion blocking action, tubocurarine might have been expected to act like hexamethonium. However, in the brain, tubocurarine produces strong long-lasting excitation thought to be due, at least partly, to disinhibition, i.e., antagonizing the action of GABA released from central inhibitory neurones. If this were the mechanism of its vasopressin releasing property other GABA antagonists, like picrotoxin, bicuculline and leptazol should have the same effect, and so they had. With regard to their other central actions, picrotoxin and bicuculline were found to be more, and leptazol less potent than tubocurarine; the leptazol effect was also of much shorter duration. The same differences seem to apply to their vasopressin releasing property. On topical application through the perspex rings, picrotoxin and bicuculline were effective in concentrations of 2 mg/ml., leptazol of 50 mg/ml. With leptazol the release ceased shortly after its removal, but continued when left inside the rings. Strychnine, an inhibitor of glycine, also released vasopressin on application to the nicotine sensitive region in concentrations of 5–10 mg/ml.

It is suggested that vasopressin release in the body is continuously inhibited by inhibitory neurones in the medulla which release GABA and glycine.

M.R.E.S. is supported by a Wellcome Research Grant.

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# Morphine selectively blocks dopamine-stimulated cyclic AMP formation in rat neostriatal slices

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Many of the varied effects of the opiate narcotics may be due to a primary interaction with cyclic nucleotide systems. In both rat brain and cultured cells, morphine markedly affects both cyclic AMP and cyclic GMP metabolism (Minneman & Iversen, 1976). Behavioural and neurochemical evidence suggests that morphine may antagonize the effects of dopamine *in vivo*, in a manner differing significantly from neuroleptic drugs (Kuschinsky & Hornykiewicz, 1972). We report here a potent inhibitory effect of morphine on dopamine-stimulated cyclic AMP formation in intact slices of rat neostriatum.

Slices (260 × 260 µm) were prepared from rat neostriata as described previously (Minneman & Iversen, 1976). Drugs and preincubated tissue were added to a final volume of 250 µl medium and incubated for 15 min with no added phosphodiesterase inhibitor. The reaction was stopped by boiling and the

**Table 1** Effect of morphine on agonist-stimulated cAMP production in rat neostriatal slices

| | *cAMP (pmol/mg protein)* | *% Basal level* |
|---|---|---|
| Basal | 3.08 ± 0.07 | 100 |
| 1 µM Morphine | 2.18 ± 0.42 | 71 |
| 10 µM Isoprenaline | 8.44 ± 0.30 | 274 |
| 10 µM Isoprenaline + 1 µM Morphine | 8.50 ± 0.15 | 276 |
| 100 µM Adenosine | 6.88 ± 0.61 | 223 |
| 100 µM Adenosine + 1 µM Morphine | 7.12 ± 0.62 | 231 |
| 10 µM Prostaglandin $E_1$ | 9.26 ± 0.52 | 301 |
| 10 µM Prostaglandin $E_1$ × 1 µM Morphine | 9.08 ± 0.31 | 295 |
| 100 µM Dopamine | 6.32 ± 0.21 | 205 |
| 100 µM Dopamine × 1 µM Morphine | 3.15 ± 0.12 | 102 |

cyclic AMP content of the supernatant determined as described previously.

In confirmation of the results of Forn, Krueger & Greengard, (1974) both dopamine and the β-adrenoceptor agonist isoprenaline caused increases in cyclic AMP formation in striatal slices (Table 1). Adenosine and prostaglandin $E_1$ ($PGE_1$) also both caused cyclic AMP increases (Table 1) and the latter two responses were not affected either by propranolol (10 µM) or by α-flupenthixol (1 µM), suggesting that there are several pharmacologically distinct receptors regulating cyclic AMP formation in rat neostriatum.

Morphine at concentrations from $10^{-7}$ to $10^{-4}$ M, caused a 30–50% decrease in cyclic AMP levels in striatal slices, however up to a concentration of $10^{-4}$ M, morphine did not affect basal adenylate cyclase activity in striatal homogenates.

Morphine ($10^{-6}$ M) also caused a complete inhibition of the dopamine-stimulated cyclic AMP levels in striatal slices (Table 1). On the other hand, morphine up to a concentration of $10^{-3}$ M did not significantly affect the increases in cyclic AMP elicited by isoprenaline, adenosine, or $PGE_1$ (Table 1), suggesting that the opiate effect in the striatum is selective for dopamine. Morphine did not inhibit the dopamine-sensitive adenylate cyclase in striatal homogenates, except at very high concentrations ($10^{-3}$M). The effect of morphine, therefore, appears to require intact cells.

The effect of morphine on both basal and dopamine-stimulated cyclic AMP levels in striatal slices was blocked by naloxone (1 µM), suggesting that it is mediated through specific opiate receptors.

These results may provide a biochemical basis for the observed antagonistic effects of opiates on dopamine receptor-mediated behaviour *in vivo*.

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## *In vitro* studies on the inhibition of monoamine uptake by Org 6582

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*In vivo* studies have revealed that Org 6582 (dl-8-chloro-11-antiamino-benzo-(b)-bicyclo[3.3.1] nona-3,6a (10a) diene hydrochloride) is a potent long acting inhibitor of rat brain 5-hydroxytryptamine (5-HT) uptake. In contrast to its effect on 5-HT uptake Org 6582 does not inhibit the *in vivo* central uptake of noradrenaline (NA) and dopamine (DA) (Goodlet, Mireylees & Sugrue, 1976; Sugrue, Goodlet & Mireylees, 1976). The objective of this study was to investigate the effects of Org 6582 on monoamine uptake *in vitro*. Drug effects on the uptake of [$^3$H]-NA and [$^3$H]-5-HT were studied using a crude synaptosomal fraction obtained from rat hypothalamus (Horn & Snyder, 1972). Rat striatal synaptosomes were employed for [$^3$H]-DA studies. In all experiments the final concentration of monoamine in the incubating medium was $2.6 \times 10^{-8}$ M. To investigate the effects of drug pretreatment on rat hypothalamic synaptosomal [$^3$H]-5-HT uptake, drugs were injected i.p. 1 h prior to sacrifice. Org 6582 and fluoxetine were equipotent in blocking [$^3$H]-5-HT uptake and both compounds were more potent than chlorimipramine. One and two days after pretreatment with Org 6582 (20 mg/kg, i.p.), [$^3$H]-5-HT uptake was inhibited 55% and 34% respectively. There was no inhibition of [$^3$H]-5-HT uptake one day after chlorimipramine (80 mg/kg, i.p.) pretreatment.

Pretreatment with Org 6582 (80 mg/kg, i.p.) had no effect on [$^3$H]-NA uptake whereas desipramine (40 g/kg, i.p.) produced a 61% inhibition after 1 hour. [$^3$H]-DA uptake into striatal homogenates was unaffected by Org 6582 (80 mg/kg, i.p.) pretreatment whilst 1 h pretreatment with benztropine (40 mg/kg, i.p.), nomifensine (30 mg/kg, i.p.) or mazindol (40 mg/kg, i.p.) inhibited uptake by 25%, 38% and 46% respectively.

In other experiments drugs were added to the incubation medium at the commencement of the 10 min preincubation period prior to addition of $^3$H-monoamine. Results are expressed as IC$_{50}$ values, which is defined as the molar concentration of drug causing a 50% inhibition of uptake. IC$_{50}$ values for blockade of [$^3$H]-5-HT, [$^3$H]-NA and [$^3$H]-DA uptake by Org 6582 were $1.8 \times 10^{-7}$ M, $2.9 \times 10^{-6}$ M and $1.3 \times 10^{-5}$M respectively. The corresponding values for chlorimipramine were $7.9 \times 10^{-9}$ M, $1.1 \times 10^{-7}$ M and $2.2 \times 10^{-6}$ M. Kinetic analysis of the inhibition of [$^3$H]-5-HT uptake by rat hypothalamic synaptosomes showed that Org 6582 was a competitive inhibitor of 5-HT uptake with a K$_i$ value of $8.9 \times 10^{-8}$ M.

These observations reveal that Org 6582 is a competitive inhibitor of 5-HT uptake and confirm those previously found *in vivo* which demonstrated that Org 6582 is a potent, long acting, selective inhibitor of 5-HT uptake.

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# Effects of intrahypothalamic injections of noradrenaline and carbachol on core temperature of unrestrained rats

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Amines (e.g. noradrenaline and acetylcholine) acting on the preoptic/anterior hypothalamic area play an important role in thermoregulation. Thus in rats, their injection into this site produces marked changes in body temperature. However, whereas some authors report hypothermia after noradrenaline, acetylcholine and their congeners, others obtain hyperthermia (e.g. Avery, 1971; Beckman, 1970; Kirkpatrick, Lomax & Jenden, 1967). These opposite effects may in part be attributed to differences in injected volume, ambient temperature and the site from which temperature is recorded (e.g. brain, rectal and liver temperatures may vary independently of each other). Since restraint, necessary for rectal temperature measurements, alone elevates body temperature, the effects of intrahypothalamic injections of the above amines were investigated in unrestrained rats in which body temperature was continuously measured from a thermistor implanted into the thoracic cavity (Poole & Stephenson, 1977a); in some rats, electrodes for recording electromyographic activity were placed in the neck muscles and an arterial cannula, for monitoring blood pressure and heart rate, in a carotid artery. Experiments were performed at 23 ± 1°C, the mid-point of their thermoneutral range (Poole & Stephenson, 1977b).

(–)-Noradrenaline hydrochloride (0.2 to 20 µg base in 1 µl of pyrogen-free 0.9% w/v NaCl solution) lowered core temperature. Latency to onset (0.8 ± 0.2 to 1.1 ± 0.1 min, mean ± s.d.) and the maximum fall (0.4 ± 0.1 to 3.1 ± 0.3°C) were dose-related, the larger doses producing correspondingly greater effects of shorter latency. Recovery occurred 18.7 ± 1.6 to 94.8 ± 13.9 min later, depending on dose, and after doses of 10 µg or less, this was followed by a 'rebound' hyperthermia of between 0.2 and 0.8°C. After small doses of noradrenaline (0.5 µg or less), this 'rebound' hyperthermia was sometimes greater than the hypothermia. Tail temperature – normally 1–2°C above ambient temperature – rose by 1.5 ± 1.2°C after noradrenaline (0.2 µg) and 2.9 ± 1.0°C after noradrenaline (10 µg), indicating marked vasodilatation and increased heat loss. Carbamylcholine hydrochloride (0.1 to 1.0 µg base in 1 µl of pyrogen-free 0.9% w/v NaCl solution) although more potent than noradrenaline produced essentially similar effects on temperature with respect to latency (0.7 ± 0.4 to 1 ± 0.5 min), fall (1.4 ± 0.3 to 2.4 ± 0.4°C) and duration (33.8 ± 8.5 to 80.5 ± 26.7 min).

The relation of the above changes in core temperature to effects on some behavioural, metabolic and cardiovascular activities will be discussed.

This work, which was supported by a grant from the Medical Research Council, forms part of S. Poole's Ph.D. Thesis, University of London.

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# Noradrenaline concentration in hypothalamic and brain stem nuclei of renovascular hypertensive rats

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Catecholamine-containing neurones in brain stem and hypothalamus, are involved in blood pressure regulation. Changes in catecholamine concentration or the activity of synthetic enzymes have been described recently in localised areas of brain stem in spontaneously hypertensive rats (Versteeg, Palkovits, van der Gugten, Wijnen, Smeets & de Jong, 1976) and deoxycorticosterone-saline hypertension (Saavedra, Grobecker & Axelrod, 1976). In the present study noradrenaline levels were examined during the development of renovascular hypertension in the rat, which was produced by applying a silver clip to one renal artery with contralateral nephrectomy (One kidney Goldblatt model). Results were compared to those obtained from sham operated litter mates sacrificed at the same time. Nuclei were isolated and removed by the micro-dissection technique of Palkovits (1973) and noradrenaline concentration estimated by the radioenzymatic method of Henry, Starman, Johnson & Williams (1973).

The mean arterial pressure (MAP) of the clipped animals 72 h after operation was 146.77 ± 2.6, compared with 119 ± 2.18 ($P < 0.01$) in the sham operated group. In all regions investigated, both in brain stem and hypothalamus, there was a reduction in noradrenaline concentration in the hypertensive animals, compared with the sham operated controls. The reduction was significant in the nucleus of the solitary tract ($P < 0.05$) and the lateral reticular nucleus ($P < 0.01$) of the brain stem, whose levels were reduced to 49.6 ± 12.7%, and 52.49 ± 6.59% respectively. In the hypothalamus, the fall in noradrenaline was significant in the anterior hypothalamic, the paraventricular and the posterior hypothalamic nuclei ($P < 0.05$). The noradrenaline concentration was reduced to 62.73 ± 10.07%, 58.11 ± 7.89% and 57.67 ± 5.49% respectively in these areas.

Seven days after operation, the MAP of the renal artery clipped rats was 162.25 ± 4.43, compared with 111.94 ± 4.54 in shams ($P < 0.01$). At this time the noradrenaline levels of all the regions investigated were not different from sham operated animals. Four weeks after operation, the MAP was 161.28 ± 6.49 in hypertensives, compared with 124.4 ± 1.78 in shams ($P < 0.01$). There was a significant change in noredrenaline concentration ($P < 0.05$) in only two regions: the parahypoglossal nucleus of the brain stem, where it was reduced to 61.93 ± 5.1% of shams, and in punches removed from the cerebellar cortex, where levels were raised to 167.08 ± 19.47%.

At present it is not clear if the early changes in monoamine concentration reported have any direct causal relationship to the rise in arterial pressure, or merely reflect secondary attempts of arterial baroreflex mechanisms to compensate for the hypertension. In addition, more dynamic measures of noradrenaline turnover will be necessary to determine whether the fall in tissue noradrenaline reflects increased or decreased activity of these central neurones.

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## The action of amino acids on evoked responses in the frog optic tectum

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The frog has a relatively simple and accessible visual system which has been subject to detailed physiological studies (Scalia, Knapp, Halpern & Riss, 1968; Gaze, 1958). The morphology of the nerve fibres within the optic tectum and their response to light falling on the retina are also documented (Potter, 1969; Maturana, Lettvin, McCulloch & Pitts, 1960), but despite this information, little is known of the synaptic transmitters involved in the anuran optic tectum. We are therefore investigating the pharmacology of the tectal response in frogs to optic nerve stimulation in the hope that this may provide information relevant to the less accessible visual pathways of mammals.

Decerebrate *Rana temporaria*, paralyzed with (+)-tubocurarine (0.2 mg i.p.) and cooled to 10°C were used. The optic nerve was electrically stimulated (0.8 ms square waves, 0.3 Hz) and the field potentials evoked in the contralateral tectum were recorded with surface electrodes and displayed on an oscilloscope and analogue pen recorder before and after averaging. The field potential complex consists of two negative waves (amplitudes 0.2–1.4 mV) with modal latencies of 12 and 30 milliseconds. The effects of possible neurotransmitter and related compounds perfused across the tectal surface on the amplitudes of these negative waves were investigated as a basis for subsequent experiments in which unitary responses will be studied.

Classified by their effect on the tectal field potentials, amino acids were found to fall into three groups:

1. no effect up to a concentration of 5 mM;
2. depression of the first wave and potentiation of the second wave;
3. depression of the entire field potential complex.

No amino acid was found which potentiated the first wave. $\alpha$-amino n-butyrate and $\alpha$-amino isobutyrate had no effect. Glycine (400 µM), taurine (400 µM) and $\beta$-alanine, the latter at high concentration (2 mM), clearly fell into the second category. The responses to glycine and taurine (400 µM) but not GABA (1 mM) were abolished by 0.1 µM strychnine. GABA caused depression of both waves of the field potential and the response was antagonised by 0.3 µM picrotoxin. Glutamate (200 µM), aspartate (200 µM) and L-homocysteate (10 µM) caused a profound, long lasting depression of the evoked tectal response with slow recovery, consistent with a non-specific excitatory action of these compounds (van Harreveld, 1959).

The relative potencies of the amino acids used may reflect the effectiveness of drug penetration or of individual uptake systems but the differential action of glycine and taurine on the two waves of the field potential response must reflect fundamental differences in the mechanism of production of the two evoked waves. As GABA equally depresses both waves, these experiments suggest that GABA may have a widespread inhibitory role in the tectum whereas glycine and taurine have selective roles at only a limited number of synapses.

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## Specific *in vivo* autoradiographic localization of [$^{3}$H]-$\beta$-alanine uptake sites in macro- as opposed to microglial cells

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Recently Schon & Kelly (1974) showed the GABA analogue [$^{3}$H]-$\beta$-alanine to be a substrate for the high affinity GABA uptake system of glial cells in the peripheral nervous system. In autoradiographic studies [$^{3}$H]-$\beta$-alanine also proved to be a selective marker for glial cells in slices of cerebral cortex (Schon & Kelly, 1975), but not for the nerve terminals associated with [$^{3}$H]-GABA uptake. We have, therefore, examined the possibility that [$^{3}$H]-$\beta$-alanine might be used *in vivo* as a specific marker for glial cells throughout the CNS. Small volumes of [$^{3}$H]-$\beta$-alanine (1 µl containing 0.41 nmol and 15 µCi) were microinjected under pressure through a fine glass microelectrode directly into the cerebellar and cerebral cortices and into the cuneate and facial nuclei. In order to prevent the catabolism of [$^{3}$H]-$\beta$-alanine by GABA: glutamate transaminase the animals were pretreated with amino-oxyacetic acid (20 mg/kg). The animals were killed within 15 min by perfusion fixation with glutaraldehyde 5%. Electron microscope autoradiographs showed high densities of silver grains evoked by [$^{3}$H]-$\beta$-alanine over both oligodendrocytes and astrocytes. No silver grains lay over adjacent nerve terminals, neuronal cell bodies or pericytes and endothelial cells lying within the basement membrane of blood vessels.

Following the proliferation of macroglia and microglia caused by either anterograde or retrograde lesions of afferent or efferent pathways, the 'reactive' astrocytes and oligodendrocytes were densely labelled by [$^{3}$H]-$\beta$-alanine. However, no silver grains were found over microglia or transitional cells lying in the vicinity of labelled macroglia. The failure of the microglial transitional cells and pericytes to accumulate [$^{3}$H]-$\beta$-alanine may be in keeping with the view that these cells are of mesodermal origin.

F.D. is indebted to the Wellcome Trust for financial support.

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## Potentiation of $\gamma$-aminobutyric acid (GABA) action by inhibiting neuroglial uptake

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Specific transport systems for the inhibitory neurotransmitter GABA exist in nerve terminals and in neuroglial cells (Iverson & Kelly, 1975). It seems reasonable to suppose that these play some role in the clearance of extracellular GABA. However, since tracer studies have not established the appropriate degree of net uptake at low substrate concentrations, the clearance function of the carrier has been questioned (Levi & Raiteri, 1974).

Sympathetic neurones are depolarized by GABA and the surrounding neuroglial cells possess a GABA carrier (see Bowery, Brown, Collins, Galvan, Marsh & Yamini, 1976). This juxtaposition has allowed a fairly direct assessment of the clearance capacity of this carrier, by observing whether the depolarizing action of GABA is indeed affected when inward carrier-mediated transport by the glial cells is inhibited.

The experiments were performed on isolated, desheathed rat superior cervical ganglia, superfused with Krebs' solution at 25°C as described previously (Bowery *et al.*, 1976). Depolarizing responses to GABA and 3-aminopropanesulphonic acid (3-APS—a potent GABA receptor agonist with very low affinity for the carrier) were recorded. Inward transport of GABA was inhibited by reducing external [Na$^{+}$] or by adding 1 mM ($\pm$)-nipecotic acid (Krogsgaard-Larsen & Johnston, 1975) or 1 mM $\beta$-aminobutyric acid (Bowery *et al.*, 1976) as false substrates.

These procedures clearly enhanced the depolarizing responses to low ($\leqslant$10 µM) concentrations of GABA but did not materially affect the equivalent response to 3-APS. For example, the depolarization produced by 3 µM GABA was increased 2–3 times during carrier-inhibition: the rate of uptake of 3 µM $^{3}$H-GABA was reduced 35–90% under these conditions. From dose-response curves in normal and low Na$^{+}$ media, it was estimated that glial transport reduced the apparent interstitial GABA concentration from 3 µM to 0.6 µM or from 10 µM to 3 µM.

Thus the glial transport system in the ganglion is capable of substantial net clearance of extracellular GABA at a concentration within the range of the 'high-affinity' transport system ($K_T \sim 7\ \mu M$). Further, Curtis, Game & Lodge (1976) have recently obtained electrophysiological evidence for potentiation of iontophoretically-applied GABA in the central nervous system by inhibiting both neuronal and glial carriers. This does not of course imply that carrier-mediated transport affects the action of synaptically-released GABA.

M.G. is in receipt of an M.R.C. studentship. This research was assisted by grants from the Medical Research Council and the Peel Medical Research Trust.

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# Inhibition of substance P release from the isolated rat substantia nigra by GABA

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The undecapeptide substance P is widely distributed within the rat central nervous system and highest levels are found in the substantia nigra (Kanazawa & Jessell, 1976). In addition biochemical and immunohistochemical studies have indicated the presence of substance P containing fibres in striato-nigral and pallido-nigral pathways in the rat brain (Kanazawa, Emson & Cuello, 1976). Using a sensitive radioimmunoassay we have previously demonstrated the potassium-evoked and calcium-sensitive release of endogenous substance P from superfused slices of rat hypothalamus (Jessell, Iversen & Kanazawa, 1976), and in the present study we have refined this technique to investigate the release of substance P from the isolated rat substantia nigra.

Substantia nigra tissue from two rats (10–12 mg) was dissected from 0.8 mm thick coronal sections of the mesencephalon and chopped at 0.2 mm intervals in two directions. Nigral slices were superfused at 37°C with Krebs bicarbonate containing 0.5% albumin at a rate of 375 μl/min. Superfusate samples were collected at 1 min intervals and substance-P like immunoreactivity in each sample, and in the nigral tissue recovered after superfusion was determined by radioimmunoassay. After 5 min of superfusion the spontaneous efflux of substance P remained constant ($8.40 \pm 0.31$ fmol $mg^{-1}$ $min^{-1}$, mean ± s.e. mean $n = 4$) and represented approximately 0.5% of tissue stores released per minute. Raising the potassium concentration in the superfusing medium to 47 mM for 2 min evoked $39.03 \pm 4.04$ fmol/mg (mean ± s.e. mean $n = 4$) increase in substance P release. Furthermore, the potassium-evoked release of substance P from the rat substantia nigra was calcium-dependent and increased as a function of the $Ca^{2+}$ concentration over the range of 0.1 to 3.0 mM $Ca^{2+}$.

In addition to the substance P pathway described, there is also strong evidence for a descending GABA mediated projection from the corpus striatum to the substantia nigra, although the synaptic connections of GABA-releasing neurones within the substantia nigra are unknown (Dray & Straughan, 1976). Superfusion of substantia nigra slices with Krebs bicarbonate containing GABA ($5 \times 10^{-5}$ M) inhibited the potassium-evoked release of substance P by $77.6 \pm 12.0\%$ (mean ± s.e. mean $n = 8$). The inhibitory effect of GABA could be reversed by the addition to the superfusion medium of picrotoxin ($5 \times 10^{-5}$ M), a GABA receptor antagonist. Superfusion with Krebs bicarbonate containing picrotoxin ($5 \times 10^{-5}$ M) in the absence of GABA did not affect the spontaneous or potassium-evoked release of substance P. It is likely, therefore, that GABA-containing neurones exert an inhibitory effect on substance P terminals within the substantia nigra, although the mechanism of this inhibition remains to be clarified.

T.J. is in receipt of a MRC studentship.

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# Preliminary investigation of a process of desensitization on mammalian presynaptic $\gamma$-aminobutyric (GABA) receptors

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There is reason to believe that presynaptic inhibition of primary afferent terminals is mediated by a chloride-dependent effect of GABA (Barker, Nicoll & Padjen, 1975; Davidson & Simpson, 1976; Gmelin, 1976; Obata, 1976). Since this action of GABA appears to be identical to that on the neurones of the sympathetic and dorsal root ganglion (Nishi, Minota & Karczmar, 1974; Feltz & Raminsky, 1974; Adams & Brown, 1975; Deschenes, Feltz & Lamour, 1976) we have recorded the changes in membrane potential and conductance evoked by GABA on single neurones of the rat dorsal root ganglion *in situ* (pentobarbitone anaesthesia, 40 mg/kg, i.p.) with double-barrelled, intracellular microelectrodes filled with K$^+$ acetate or sulphate.

Typically, the response of the ganglionic neurones to GABA was a biphasic depolarization. Brief applications of GABA resulted in a transient depolarization whose peak amplitude ($\leqslant 25$ mV) and time to peak (2–5 ms) was dependent on the membrane potential (reversal level $-40$ mV) and the magnitude of the GABA application. The response to GABA was saturable and towards the end of the application the decaying depolarization was interrupted by a hump. When GABA ($10^{-5}$ to $10^{-3}$ M) was added to the superfusate or the iontophoretic pulses of GABA (20–100 nA, 1 s duration) were repeated more often than 0.1 Hz or their duration extended to 10 s, the initial depolarization decayed to a plateau level, which was maintained at about one-third of the initial level for the remainder of the GABA application. K$^+$-sensitive electrodes showed these changes in potential to be accompanied by an increase in the extracellular K$^+$ concentration. During action potentials this increase in the extracellular K$^+$ was also apparent from a decrease in the amplitude of the hyperpolarizing after-potentials.

When the ganglion was superfused with GABA ($10^{-4}$ to $10^{-3}$ M), the depolarizing responses to brief iontophoretic pulses of GABA applied at a distance of 30–80 µm from the soma during the plateau phase of the maintained depolarization, were reduced in amplitude. Since this loss in GABA sensitivity could not be restored by hyperpolarizing the membrane and was accompanied by a reduction in the increased Cl$^-$ conductance normally associated with the response, the desensitization was not attributable to a change in equilibrium level of the ions involved.

With financial support of the D.G.R.S.T. (73–1125) and of the C.N.R.S. (ERA 237). M.D. was a Canadian MRC-CRSQ postdoctoral fellow: present address: Département de Physiologie, Faculté de Médecine, Université Laval, Quebec C1K 7DA Canada. B. R–D holds from the D.G.R.S.T. and I.A.M.O.V. training program fellowship.

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# Efflux characteristics of isotopically labelled $\gamma$-aminobutyric acid (GABA) and L-glutamate in the rat cuneate nucleus

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The experiments reported here are part of a series designed to investigate the efflux characteristics of isotopically labelled putative neurotransmitters in the *in vivo* superfused rat cuneate nucleus. Details of the superfusion and isotope-labelling methods in the electrophysiologically identified cuneate nucleus of the chloralose-urethane anaesthetized rat have already been reported (Assumpção, Bernardi, Dacke & Davidson, 1976).

After a 60 min period of superfusion with a solution containing 2 µCi/ml of 1-[$^{14}$C]-GABA or L-glutamate, the pial surface of the exposed cuneate nucleus was superfused with artificial cerebrospinal fluid (Merlis, 1940) at 62.5 µl/min and 5 min fractions collected for estimation of radioactivity by liquid scintillation spectrometry. The results, when plotted semi-logarithmically against time, with computed lines of best fit, revealed multi-phase spontaneous effluxes for both GABA (Figure 1) and glutamate.

In some experiments the superfusate was changed to one containing elevated (40 mM) K$^+$ for one or more 10 min periods. This caused significant ($P<0.01$) increases in the efflux of labelled GABA which could be measured by the departure of the observed points from those predicted by the line of best fit computed from the remaining points in that particular phase (Figure 1). Roberts (1974) measured evoked GABA release from the dorsal column nuclei as a percentage increase in isotope efflux over spontaneous release, but it is clear from Figure 1 that a percentage measurement may not be particularly meaningful unless it is also known at which phase in the efflux it is calculated.

Interestingly, although GABA and glutamate are respectively the major inhibitory and excitatory transmitter candidates in the cuneate nucleus (for review see Davidson, 1976), we have obtained no evidence for a potassium evoked increase in glutamate efflux such as reported by Roberts (1974). The reason for this discrepancy in our results is not clear at present.

JAA and NB are supported by a grant from the Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil.

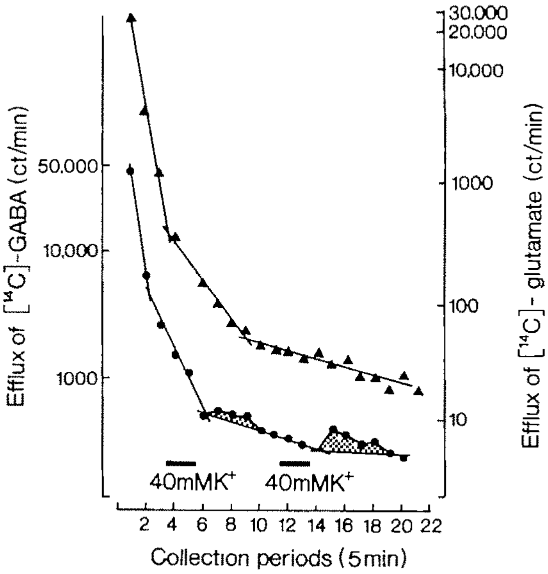

**Figure 1** Effect of high potassium stimulation on the efflux of 1-[$^{14}$C]-glutamate (upper graph, ▲) and GABA (lower graph, ●) from the superfused cuneate nucleus of rats pretreated with amino-oxyacetic acid (20 mg/kg, i.p.). The ordinate for the upper graph is on the right, for the lower graph on the left. The abscissa indicates the number of 5 min fractions collected in each experiment; horizontal bars indicate 10 min periods of stimulation with high (40 mM) K$^+$ C.S.F. The hatched areas in the second and third phases on the lower graph indicate the K$^+$-evoked GABA efflux as an increase in sample radioactivity compared with that predicted from the least squares line of best fit computed from the remaining points in the phase. No corresponding increase in glutamate efflux can be detected in the upper graph.

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# Antagonism of synaptic inhibition in the rat substantia nigra by tetanus toxin

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It is known that tetanus toxin blocks strychnine-sensitive postsynaptic inhibition in the spinal cord (Brooks, Curtis & Eccles, 1957). This action of the toxin is believed to be due to a reduced release of the inhibitory transmitter glycine (Curtis & De Groat, 1968). Recently, it has been suggested that the toxin also reduces the release of GABA since it blocks bicuculline-sensitive inhibition in the spinal cord and cerebellum (Curtis, Felix, Game & McCulloch, 1973). In the present experiments the effects of tetanus toxin were investigated on caudate evoked post-synaptic inhibition in the substantia nigra where there is good evidence that GABA is the inhibitory transmitter (see Dray, Gonye & Oakley, 1976).

Experiments were performed on rats anaesthetized with urethane (1.2–1.4 g/kg, i.p.). Extracellular recordings were made from single nigral neurones from the centre barrel (4 M NaCl) of a 7 barrel microelectrode. The following substances were ejected from the outer barrels using standard microelectrophoretic techniques: Acetylcholine Cl (1 M), GABA (0.5 M pH 3.5), dopamine HCl (DA 0.5 M), 5-hydroxytryptamine bimaleate (5-HT 0.5 M) bicuculline methochloride (BMC 0.005 M in 0.165 M NaCl), DL-homocysteate (DLH 0.2 M pH 7), tetanus toxin ($1.5 \times 10^2$ mouse MLD in 0.165 M NaCl). Tetanus toxin (Burroughs Wellcome) was administered either via a micrometer syringe and a glass pipette (tip dia. 20–30 μ) attached to the multibarrel electrode such that the latter projected 500–800 μM beyond the toxin pipette or by microelectrophoresis from one barrel of the microelectrode. Postsynaptic inhibition was evoked on substantia nigra neurones by single 100 μA pulses ($2\ s^{-1}$, 100–300 μs) via a bipolar stimulating electrode positioned in the ipsilateral caudate nucleus.

Single microinjections (0.5–1 μl) of $10^2$–$10^3$ mouse MLD of toxin abolished synaptic inhibition evoked in 10 nigral neurones within 4–7 minutes. The toxin had no discernible effect on spontaneous firing rates or on responses induced by ejections of DLH, GABA, DA or 5-HT. By contrast, BMC (20–50 nA for 2–25 min) caused 50–100% antagonism of synaptic inhibition on 7 neurones and simultaneously abolished responses to electrophoretically ejected GABA. Administered electrophoretically tetanus toxin was much less effective in antagonizing synaptic inhibition than when administered by microinjection. Hence, 100–200 nA toxin ejected for 45–60 min only partially reduced inhibition in 3 neurones and was without effect on 2 neurones.

These results provide further evidence that tetanus toxin antagonizes GABA mediated inhibition in the central nervous system by a presynaptic action.

This work was supported by the Medical Research Council.

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# Use of protease inhibitors to protect subcutaneously injected peptide hormones against local degradation

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Although peptide hormones are usually administered subcutaneously, the extent to which they are degraded before absorption has been insufficiently studied. Local monitoring of the injection site after giving isotopically labelled hormone indicates that radioactivity disappears rapidly (Binder, 1969), but it cannot be assumed that the labelling atoms remain within bioactive molecules. Data obtained by radioimmunoassay also requires critical evaluation because of the lack of correlation between immunological and biological activity in many peptide fragments, and few bioassays are sufficiently sensitive to follow blood levels.

We have studied the effect of protease inhibitors on local degradation, using bovine parathyroid hormone (bPTH 1–84) and synthetic amino-terminal fragments of the bovine and human sequences (bPTH 1–34 and hPTH 1–34) (Tregear, Reitschoten, Greene, Keutmann, Niall, Reit, Parsons & Potts, 1973; Niall, Sauer, Jacobs, Keutmann, Segre, O'Riordan, Aurbach & Potts, 1974) in the subcutaneous version of a bioassay depending on measurements of hypercalcaemia in chicks (Parsons, Reit & Robinson, 1973). Both Trasylol and ϵ-amino caproic acid (EACA) substantially enhanced the hypercalcaemia measured 2 h after injection. It was confirmed by radioimmunoassay that this enhancement reflected an increase in circulating blood levels of hPTH 1–34, the greatest increase being seen 1 h after injection. Similar enhancement in circulating blood levels of another peptide hormone was observed when EACA and Trasylol were added to porcine calcitonin, injected subcutaneously to chicks. Large doses of calcitonin can be given to these birds and the resulting blood levels were followed directly by injecting their plasma intravenously to rats and measuring the hypocalcaemic response as described by Kumar, Slack, Edwards, Soliman, Baghdiantz, Foster and MacIntyre (1965).

A quite independent approach to the evaluation of subcutaneous losses appears to be possible by using controlled intravenous infusion to imitate the changing blood levels measured after s.c. injection. The intravenous technique allows certainty that all hormone administered enters the bloodstream and comparisons of s.c. and i.v. administration in freely mobile dogs with indwelling venous cannulae have yielded preliminary evidence to confirm that substantial local destruction must occur when hPTH 1–34 is injected subcutaneously.

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## Drug clearance in the rabbit twenty-four hours after an intoxicating dose of ethanol

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Acute ethanol intoxication is known to inhibit the *in vivo* metabolism of a variety of drugs, including barbiturates, in animals and in man (Rubin, Gang, Misrad & Lieber, 1970), although it is not so clear whether this is a direct inhibitory effect of the ethanol itself. The levels of ethanol required to inhibit the metabolism of most drugs by the hepatic microsomal fraction, around 100 mM (Rubin *et al.*, 1970; Cinti, Grundin & Orrenius, 1973), are in general higher than those encountered except under cases of severe intoxication. It was of interest therefore to examine *in vivo* drug clearances several hours after a single dose of ethanol, when the ethanol had been completely removed from the blood.

Rabbits were fed ethanol (4.25 g/kg) as a 50% solution (v/v) by stomach tube. A peak blood ethanol concentration (mean ± s.e. mean, $n = 3$) of 397 ± 7 mg/100 ml was achieved within 2 h and had fallen to 1.3 ± 0.9 mg/100 ml by 24 hours. Blood acetaldehyde levels after 24 h, 0.11 ± 0.01 mg/100 ml, were no different from control values 0.09 ± 0.01 mg/100 ml ($P > 0.05$). Hexobarbitone sodium (50 mg/kg) administered intravenously, was removed from the blood with a half life (mean ± s.e. mean, $n = 7$) of 22.9 ± 1.3 min in control animals and 41.0 ± 5.8 min ($n = 4$, $P < 0.01$) in animals pretreated with ethanol 24 h previously. There appeared to be no significant difference in the half life for the removal of intravenously administered aniline hydrochloride (50 mg/kg) between control 29.5 ± 1.2 min ($n = 6$) and ethanol pretreated animals 34.1 ± 2.6 min ($n = 4$, $P > 0.05$). The decrease in the rate of removal of hexobarbitone could not be accounted for by any change in the hepatic microsomal metabolism of hexobarbitone measured *in vitro*, control 2.3 ± 0.1, ethanol pretreated 2.2 ± 0.1 nmol min$^{-1}$ mg protein$^{-1}$ ($n = 7$, $P > 0.05$) or in the levels of microsomal cytochrome P-450, control 1.0 ± 0.1 ethanol pretreated 1.0 ± 0.1 nmol mg protein$^{-1}$ ($n = 3$). Microsomal aniline hydroxylation was similarly unchanged, control $0.50 \pm 0.02$, ethanol pretreated $0.57 \pm 0.06$ nmol min$^{-1}$ mg protein$^{-1}$ ($n = 7$, $P > 0.05$).

One possible explanation for the decrease in the rate of hexobarbitone removal might lie in the release of adrenal cortical hormones by the ethanol. Serum cortisol levels were $4.2 \pm 0.5$ µg/100 ml ($n = 5$) in control animals and $14.5 \pm 2.4$ µg/100 ml ($n = 6$, $P < 0.01$) in animals pretreated with ethanol 24 h previously. Corticosterone administered to rats *in vivo* is known to inhibit the hepatic microsomal metabolism of hexobarbitone (Chung & Brown, 1976).

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# Influence of age and sex on the duration of action of ketamine in the rat

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In order to study the influence of age and sex on the duration of action and metabolism of ketamine, groups of six rats of both sexes, ranging in age from one to 16 weeks were injected intraperitoneally with ketamine hydrochloride (75 mg/kg). Time to loss of righting reflex (onset time) and duration of loss of the righting reflex (sleeping time) were recorded for each rat. The rats were killed by decapitation at the point of recovery, and blood samples were collected for assay of plasma concentrations of ketamine, its *n*-demethylated metabolite (I), and the subsequent oxidation product (II), as previously described (Livingston & Waterman, 1976).

There was a significant increase in onset time from 1 to 6 weeks of age in both sexes, but after this it did not alter significantly. Sleeping time decreased markedly with increasing age from 1 to 4 weeks, in both males (97.2 to 24.1 min) and females (90.1 to 30.0 min) but thereafter it did not vary significantly. The females tended to show a longer sleeping time than the males from 4 weeks old onwards but the difference was only statistically significant in the 16 week old age group.

In the male rats the concentration of ketamine in the plasma at recovery was $4.1 \pm 0.39$ µg/ml in the 1 week old rats, falling to $2.67 \pm 0.25$ µg/ml at 6 weeks, after which it remained close to this level. Metabolite I levels followed the same pattern ($2.27 \pm 0.26$ µg/ml at 1 week to $1.27 \pm 0.17$ µg/ml at 6 weeks), but no metabolite II was detected in the plasma until 3 weeks, the concentration then rose steadily with age to 6 weeks when it reached $1.25 \pm 0.19$ µg/ml. The changes in plasma levels of ketamine and its metabolites in the female rats followed the same pattern as the males, but the concentrations of ketamine and metabolite I were always higher than in the males of corresponding age, whilst the levels of metabolite II were always lower.

The data suggest that the decreasing sensitivity to the actions of ketamine, as demonstrated by a decrease in sleeping time from 1 to 4 weeks of age, is related to the appearance of the second metabolite in the plasma. In addition, there appears to be a sex difference in the duration of action of the drug which may be related to the difference in the ability of the sexes to produce metabolite II. These results indicate that, in the rat, the second (oxidative) step may be the rate limiting one in the degradation of this drug.

A.E.W. is a Wellcome Research Training Scholar.

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# First-pass effect of the beta-adrenoceptor blocking agent pindolol in the dog

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Pindolol ((±)-4-(2-hydroxy-2-isopropylaminopropoxy)-indole; Visken), a potent and specific beta-adrenoceptor blocking agent (Aellig, 1976a), is chemically related to all other beta-adrenoceptor blocking substances. The pharmacokinetic behaviour of the compound in man, however, has been found to be different from other beta-adrenoceptor antagonists in clinical use in that it is only partially metabolized in man and has a high systemic availability after oral administration (Johnsson & Regårdh, 1976; Meier & Nüesch, unpublished observations). Metabolism by the liver during its first passage is referred to as the first-pass effect. The major cause of the low bioavailability of most beta-adrenoceptor blocking agents is due to a high first-pass effect. Since the dog metabolizes pindolol in a similar way, but to a greater extent than man, experiments were performed to investigate the first-pass effect of the drug in the dog.

Two beagle dogs, each weighing 13 kg, were given an intravenous injection of [$^{14}$C]-pindolol labelled in the 3-position of the indole ring; the dose (25 mg) given contained 250 μCi $^{14}$C activity. After a wash-out period of 3 weeks, the same dose was administered orally. Following both doses, blood samples were taken at intervals during 48 h and urine and faeces collected for 96 h following administration. [$^{14}$C] activities (pindolol plus metabolites) were determined in blood, plasma, urine and faeces by liquid scintillation counting. Unchanged pindolol was determined in plasma and urine samples fluorimetrically (Pacha, 1969).

Absorption of pindolol from the gastrointestinal tract was found to be rapid and complete. After both oral and intravenous administration, approximately 74% of the administered [$^{14}$C] activity was excreted in the urine and 23% in the faeces during the 96 h collection period. Only 4 to 7% of the dose was excreted in the urine as unchanged pindolol. This strong metabolism of pindolol in the dog was confirmed by comparing the area under the plasma concentration versus time curve (AUC) of [$^{14}$C] activity and that of unchanged substance. These results are in contrast to those previously obtained in man in which unchanged pindolol amounted to 40% of the total dose administered. Based on the urinary excretion and the AUC's of unchanged pindolol after oral and intravenous administration the bioavailability of pindolol in the two dogs was calculated to be 54.2% and 55.2% respectively. The first-pass effect of pindolol in the dog was therefore 45%, compared to 13% in man (Meier & Nüesch, unpublished observations). These values correlate well with the degree of beta-adrenoceptor blocking activity estimated in the dog (Saameli, 1972) and man (Aellig, 1976b) following oral administration compared with that following intravenous administration.

It is concluded that, as with other beta-adrenoceptor blocking agent, the first-pass effect of pindolol is directly related to the degree of metabolism of the drug by the liver.

We thank Dr R. Voges for the synthesis of the [$^{14}$C]-labelled pindolol.

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## Absorption of nicotine by man during cigar smoking

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The absorption of nicotine during cigar smoking was studied in 7 male subjects each of whom smoked a single cigar labelled with a known amount of (2′-$^{14}$C) nicotine, range 41.3 to 45.5 μCi. The cigars were 77 mm long and similar to small commercially available varieties. The smoke transfer characteristics of exogenous radioisotopically labelled nicotine were similar to those of endogenous material (Houseman & Hopper, 1974).

Cigars were smoked at one puff per min down to a standard butt length, total 14–23 puffs. After each puff the subject exhaled into a smoke collecting mask through which air was drawn continuously. The cigar itself was mounted in a smoking cartridge designed to collect all the sidestream smoke and ash. Before smoking, a brachial artery catheter was inserted and blood samples were taken at intervals during and following the cigar. Samples were analysed for nicotine and cotinine using a radiochemical method (Turner, 1971).

The cigars contained 16 mg nicotine in total. An average of 33% of the total nicotine was taken into the smoker's mouth as mainstream smoke and was thus available for absorption, a higher proportion than the 25% found previously for cigarettes (Armitage, Dollery, George, Houseman, Lewis & Turner, 1975). The proportion of the mainstream smoke nicotine which was retained varied considerably, between 36 and 97%. The average dose of nicotine retained by the 7 subjects was 2.1 mg, range 1.0 to 4.5 mg. Subjects who retained most nicotine had the highest peak arterial blood concentrations of nicotine which ranged between 8 and 64 μg/l. In two subjects the rates of rise in arterial nicotine concentrations, 1.7 and 3.8 μg $l^{-1}$ $min^{-1}$, were comparable to the rates previously measured in inhaling cigarette smokers, range 3.5 to 5.3 μg $l^{-1}$ $min^{-1}$. In other subjects the rate at which the nicotine was absorbed was less 0.4–0.7 μg $l^{-1}$ $min^{-1}$.

Nicotine in cigar smoke is thought to be absorbed mainly from the buccal mucosa, facilitated by the alkaline pH of the smoke (Armitage & Turner, 1970). This process is much less rapid than alveolar absorption following inhalation of smoke into the lung. Cigar smoke is more irritant than cigarette smoke and is thought to be inhaled only rarely. The lower mortality of exclusive cigar smokers compared with cigarette smokers supports this supposition (Hammond, 1966). However, the rapidity and completeness of nicotine absorption observed in the present study raises the possibility that some smokers can inhale cigar smoke. If habituated cigarette smokers retain the habit of inhaling smoke when they switch to smoking cigars then they may be at particular health risk since even small cigars contain more nicotine and tar than the strongest cigarette.

This study was supported by the Tobacco Research Council.

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## Effect of prenatal phenytoin treatment on postnatal development

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Adult pregnant Wistar rats were treated with phenytoin (100 mg/kg) orally from day 7–19 of pregnancy, and a control group was pair-fed during the whole treatment period. Within 24 h after parturition, the offspring were culled to eight per litter and reared by fostering or cross-fostering. The physical and behavioural development of the offspring was observed up to 90 days of age.

Prenatal phenytoin treatment reduced the fertility and litter size of the rats. There was also a reduced survival of the offspring and a reduction in body weight which persisted to the end of the experiment, though both of these effects could be reduced by cross-fostering. There was a wide variation in time of ear opening, eye opening and tooth eruption in the treated compared with the control groups. Chromodacryorrhea was present in the treated animals and remained up to age 90 days.

Certain neurological deficits were also seen in the prenatal phenytoin group. For example, there was a delay of up to 15 days in the development of the dynamic righting reflex, a decrease in ability of offspring to stay on a rotating rod, and a decrease in ability to walk along a narrow elevated path. There seemed also to be some loss of visual depth perception. However, there was no change in the development of crawling and walking activities at 9–21 days of age, and no changes were observed in a head dipping test or in a conditioned avoidance test at 26–34 days.

There was a significant decrease in the brain weight of the treated group at age 3 days which remained significantly lower than the controls even at 90 days, but no change in the brain weight/body weight ratio. There was no difference in the cerebellar DNA content.

Prenatal phenytoin treatment caused a significant decrease in the R.B.C. ghost cholinesterase activity at age 30 and 70 days, an increase in the brain and cerebellar cholinesterase at age 90 days but no significant change in plasma cholinesterase activity at 30 days of age. There was a marked increase in the serum and brain tryptophan, and brain 5 H.T. and 5 H.I.A.A. levels at 3 days of age but not at 90 days. This seemed to be related to early feeding problems.

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## Comparative teratogenicity of six antiepileptic drugs in the mouse

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Evidence has accumulated associating the birth of congenitally malformed children to epileptic women with anticonvulsant medication (Speidel & Meadow, 1972). As most epileptic patients are on multiple drug therapy it is difficult to assess which drug or combination of drugs, if any, is responsible for the defects observed.

Animal studies to date have been mainly concerned with testing phenytoin and phenobarbitone. Therefore, in the present study six commonly used antiepileptic drugs were tested in pregnant CD1 mice ($n = 16$) to see if they were teratogenic, and if so, whether they could be placed in some order of teratogenicity.

The drugs used were all given at 3, 9 and 18 times the therapeutic dose and were carbamazepine (40, 120 and 240 mg/kg), clonazepam (0.3, 0.9 and 1.8 mg/kg), ethosuximide (60, 180 and 360 mg/kg), phenobarbitone (10, 30 and 60 mg/kg), phenytoin (15, 45 and 90 mg/kg), and primidone (30, 90 and 180 mg/kg) together with carboxymethylcellulose (CMC) and absolute control groups. All drugs were suspended in 1% CMC and given by gavage on days 6–16 of pregnancy which covers the period of embryogenesis.

The main defects observed in the treated groups were cleft palate (with the exception of clonazepam) and enlarged cerebral ventricles. Other major defects such as exencephaly, exomphalos, phocomelia, open eyes, and undescended testes were seen less frequently. If the results obtained for each drug are pooled on either a per foetus or a per litter basis, the drugs can be divided into three main categories of teratogenicity.

On a per foetus basis, phenytoin is in a category of its own in that it produced the highest incidence of foetal defects (20.9%). Primidone, carbamazepine and phenobarbitone fall into the next category, the percentage of foetal defects being 4.8%, 4.7% and 4.7% respectively. In the third category are clonazepam (2.7%) and ethosuximide (3.2%) which produced the lowest incidence of malformations, which are not clearly shown in these experiments to be significantly different from the spontaneous control values of 1.3%. When assessed on a pooled litter basis (mean percentage litter effect) a similar pattern is seen.

It is interesting that similar defects, e.g. cleft palate have been reported in both the mouse and man. These results indicate that some anticonvulsants are safer than others in the mouse, and this may be true for man also. Therefore, it would be beneficial to try to determine from epidemiological surveys, which drug(s) is safest for women of child bearing age.

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## A comparison of the effects of ouabain and potassium-free Ringer on the electrogenic sodium pump and slow synaptic inhibition in bullfrog sympathetic ganglia

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Slow synaptic inhibition in sympathetic ganglia is generated by a unique mechanism that differs from the increase in postsynaptic membrane conductance found at most synapses. Two hypotheses have been advanced to explain the mechanism of generation of the slow IPSP (inhibitory postsynaptic potential). Nishi & Koketsu (1967, 1968) proposed that the slow IPSP is produced by activation of the electrogenic $Na^+$ pump. On the other hand, Weight & Padjen (1973) proposed that the slow IPSP is generated by a decrease in resting $Na^+$ conductance. To resolve this controversy, we studied the effects of ouabain and $K^+$-free Ringer on both electrogenic $Na^+$ pumping and slow synaptic inhibition. The bullfrog sympathetic ganglion was investigated using sucrose gap recording. The nicotinic depolarization produced by acetylcholine (ACh, $3 \times 10^{-3}$ M) was followed by an after-hyperpolarization which has been shown, in mammalian sympathetic ganglia, to be due to the electrogenic pumping of $Na^+$ (Brown, Brownstein & Scholfield, 1972; Lees & Wallis, 1974). This ACh after-hyperpolarization was abolished by $K^+$-free Ringer or after 1 h in ouabain ($10^{-6}$ M). In contrast to this inhibition of the $Na^+$ pump, $K^+$-free Ringer enhanced the slow IPSP. Ouabain (1 h, $10^{-6}$ M) reduced but did not block the slow IPSP. Even after 1 h in ouabain ($10^{-6}$ M), the $K^+$-free Ringer still potentiated the slow IPSP. These data suggest that the slow IPSP is not due to activation of the electrogenic $Na^+$ pump. On the other hand, the data are consistent with the hypothesis that the slow IPSP is generated by a decrease in resting $Na^+$ conductance.

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# Alterations in $^{22}Na$ fluxes of arterial smooth muscles of spontaneously hypertensive rats

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The rats obtained in 1963 by Okamoto & Aoki are spontaneously hypertensive (SH rats). These SH rats present an increase in vascular resistance which seems to be associated with an altered reactivity of vascular smooth muscle (Hausler & Finch, 1972) which could be due to changes in excitation or excitation-contraction coupling processes. Jones (1973, 1974) has observed an increased $K^+$ and $Na^+$ turnover. In order to confirm these findings we have analyzed $Na^+$ turnover from tail arteries obtained from male SH and normotensive Wistar (NW) rats of the same age which have been used as controls. $^{22}Na$ fluxes have been studied after equilibration of intracellular and extracellular specific activities by incubating the arteries for 90 min at 35°C gassed with 95% $O_2$ and 5% $CO_2$ in a physiological solution containing the tracer and having the following composition (mM): NaCl 120.8, KCl 5.9, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5, $NaH_2PO_4$ 1.2 and glucose 11.5. Effluxes were performed using a superfusion technique (Hamon, Papadimitriou & Worcel, 1976) at 35°C. The efflux curve can be separated into a series of exponential components among which the intermediary $Be^{-k_2t}$ corresponds to the membrane limited efflux from the smooth muscle cells (Garay, Moura & Worcel, unpublished observations). $^{22}Na$ efflux rate $k_2$ was increased in 8 weeks old SH rats as well as the smooth muscle cell $Na^+$ compartment B (Table 1). The efflux rate becomes normal at 12 and 20 weeks SH rats. The compartment B is normal at 12 weeks and smaller than in controls at 20 weeks. The action of ouabain on $^{22}Na$ efflux rate $k_2$ was studied on arteries from 8 weeks old rats in order to quantify its effects on the $Na^+$ pump and to distinguish between ouabain sensitive and insensitive fractions of $^{22}Na$ efflux.

From these experiments we could determine that ouabain sensitive efflux in completely blocked muscles is identical in NW (0.106 $min^{-1}$) and SH rats (0.099 $min^{-1}$). According to these values, it appears that the ouabain insensitive fraction is greatly increased (+63%) in SH (0.067 $min^{-1}$) as opposed to NW rats (0.041 $min^{-1}$). Surprisingly the $K_m$ constants for ouabain action on the $Na^+$ pump have been found to be different: $K_{SH} = 3.3 \times 10^{-4}$ M and $K_{NW} = 7.6 \times 10^{-4}$ M.

In conclusion at an early stage of development of hypertension there is an increase in ouabain insensitive, membrane limited $^{22}Na$ efflux from arterial smooth muscle cells. This is accompanied by an increased $Na^+$ content in the cellular compartment and an alteration in the sensitivity of the $Na^+$ pump to ouabain which is difficult to interpret at the moment.

This work was supported by grants from the I.N.S.E.R.M. and D.G.R.S.T., France.

**Table 1** $^{22}Na$ efflux from arterial smooth muscle cells of NW and SH rats

| *Age* | *8 Weeks* | | 12 Weeks | | *20 Weeks* | |
|---|---|---|---|---|---|---|
| | *NW* | *SH* | *NW* | *SH* | *NW* | *SH* |
| $k_2$ ($min^{-1}$) | 0.144±0.005(1) | 0.173*±0.012 | 0.145±0.004 | 0.153±0.004 | 0.139±0.003 | 0.143±0.004 |
| B (mmol/kg wet weight) | 2.7 ±0.09 | 3.1 *±0.12 | 3.0 ±0.15 | 2.9 ±0.16 | 3.2 ±0.17 | 2.5** ±0.14 |
| n | 7 | 6 | 7 | 9 | 10 | 11 |

(1) mean ± s.e. mean; * $P < 0.05$; ** $P < 0.01$.

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# Mechanism of action of angiotensin II on the membrane potential of rat myometrium

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Angiotensin II (Ang II) produces a contraction of visceral and vascular smooth muscle of different species (Worcel, Papadimitriou & Meyer, 1971). Since this spasmogenic action might be initiated by a change in cell membrane excitability we have attempted to study the effects of the peptide on rat myometrium membrane potential. Contraction and transmembrane potential were measured simultaneously by the double sucrose-gap method (Burnstock & Straub, 1958). Uterine strips were obtained from 220–250 g Wistar rats pretreated with diethylstilboestrol dipropionate (0.5 mg/kg) the day before the experiment. Smooth muscle strips of the longitudinal layer 3 cm long and 1 mm width were used. The solutions used were: (1) physiological solution (mmol): NaCl 120.8, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5, $NaH_2PO_4$ 1.2, glucose 11.5, gassed with 95% $O_2$ and 5% $CO_2$ or with the same solution in which $NaHCO_3$ and $NaH_2PO_4$ are replaced by Tris $Cl^-$ and gassed with 100% $O_2$. (2) KCl isotonic solution and (3) isotonic sucrose solution of a resistivity higher than 1 MΩ/cm. The experiments were performed at 35 °C. Under these conditions Ang II at $10^{-7}$M (Figure 1b) which already produces a maximal contraction (Worcel *et al.*, 1971) induced a depolarization and an increase in membrane conductance. This increase in membrane conductance is not potential dependent since it is observed even when the membrane potential is reset at the baselevel electrically. At a $10^{-6}$M concentration which produces a supramaximal contraction, Ang II maximally depolarizes the preparations (Figure 1a). When all $Na^+$ in the test solution is replaced by either $Li^+$ or $Mg^{2+}$ the depolarizing effect of $10^{-6}$M Ang II disappears. Under these conditions Ang II produces a slight initial hyperpolarization. When all $Cl^-$ is replaced by either $NO_3$- or cyclohexanesulfamate Ang II $10^{-6}$ M still produces a 20 mV depolarisation. The suppression of extracellular $Ca^{2+}$ or $K^+$ does not have any effect on the depolarizing action of Ang II, which is not changed either by ouabain $10^{-3}$ M.

In conclusion Ang II produces a depolarization of the uterine smooth muscle membrane, through an increase in the membrane conductance to $Na^+$ accompanied by a small increase in potassium conductance. These results confirm previous findings using isotope fluxes (Villamil, Nachev & Kleeman, 1970; Hamon & Worcel, 1973) but not the results of Turker, Page & Khairallah (1967) who considered that Ang II might stimulate the $Na^+$–$K^+$ pump in rat myometrium.

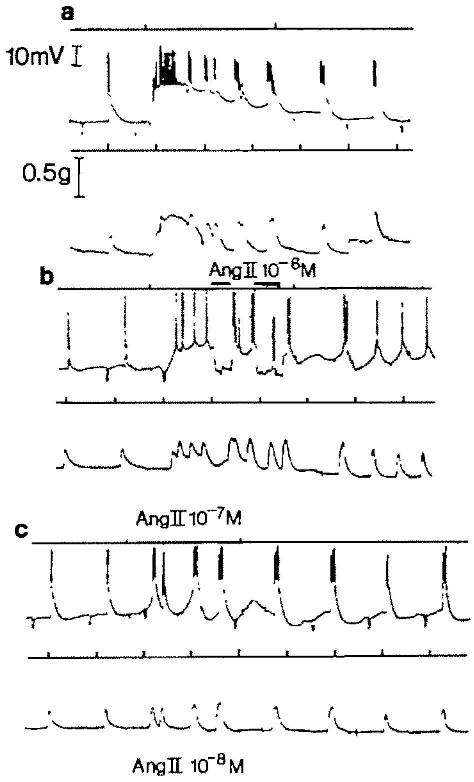

**Figure 1** Angiotensin II (Ang II) in physiological solution. (a) Effects of Ang II $10^{-6}$ M; (b) Effects of $10^{-7}$M. Electrical repolarization was performed at times marked by the horizontal bars. (c) Effects of Ang II $10^{-8}$ M. The vertical marks on the time scale indicate minute intervals.

This work has been supported by grants from the I.N.S.E.R.M. and D.G.R.S.T. (France).

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# High and low affinity ouabain binding sites in guinea-pig atria

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We have previously reported that doses of ouabain between 1 nM and 10 nM stimulate the Na pump and evoke a positive inotropic effect in guinea-pig atria, whereas doses higher than 10 nM inhibit the Na pump (Ghysel-Burton & Godfraind, 1975).

We have also reported that a specific ouabain binding represented by a Langmuir binding curve occurred likely on the sites responsible for the inhibition (Godfraind & Lesne, 1972; Ghysel-Burton & Godfraind, 1975).

The purpose of the present experiments was to examine whether these stimulatory and inhibitory effects of ouabain were related to one mode of specific binding.

Experiments were performed on isolated guinea-pig atria (resting tension 500 mg) stimulated at a rate of 3.3 Hz and bathed in Tyrode solution at 30°C (mM: NaCl 137; KCl 2.7 either 6 or 12, $CaCl_2$ 1.82, $MgCl_2$ 0.105, $NaH_2PO_4$ 0.417, $NaHCO_3$ 11.9, glucose 5.5) equilibrated with a mixture of 95% $O_2$/5% $CO_2$. [$^3$H]-ouabain was added together with [$^{14}$C]-inulin in the incubation fluid. This procedure allowed to correct tissue ouabain uptake for extracellular ouabain content.

For 6 mM KCl, within the range of ouabain concentrations comprised between 30 and $10^3$ nM, the cellular ouabain uptake showed both a linear and a saturable component and was found to fit the equation:

$$U = a\,Cm + \frac{b\,Cm}{Cm + Kb} \qquad (1)$$

where U is the tissue concentration corrected for cardiac glycoside content of inulin space, Cm is the glycoside concentration in the medium, a is the proportionality constant for the linear non-saturable uptake, b is the capacity and Kb is the equilibrium constant for the saturable binding sites (Godfraind & Lesne, 1972). Values of the constants were as follows: a (1/kg wet weight) 0.319, b (n mol/kg wet weight) 372, Kb (nM) 389.

For ouabain concentrations between 1 nM and 10 nM, the observed ouabain tissue content was higher than that expected from equation (1) using the constants given above. After subtracting the non-saturable uptake, aCm, from the observed contents, the saturable uptake showed two components, one at concentrations between 1 and 10 nM and the other at higher concentrations.

This indicates the existence of a high and of a low affinity uptake process. The high affinity process was represented by a S shaped curve with a Hill coefficient near 2; the concentration producing a 50 per cent saturation was equal to 2.5 nM. The capacity of this binding was only 3 per cent of the capacity of the low affinity binding represented by equation (1).

Both high and low affinity bindings were sensitive to change in KCl concentration. For 2.7 mM KCl, the two bindings were displaced to the left. They were displaced to the right for 12 mM KCl.

The sensitivity of the two bindings to extracellular KCl indicates that both might occur on Na pumping sites.

This work has been supported by FRSM (grant No. 20121).

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## Uptake of choline by rabbit corneal epithelium

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The corneal epithelium in most mammals has a content of acetylcholine (ACh) and choline acetyltransferase (ChAc) higher even than ganglion and brain, although its only nerve supply is sensory. ACh in this tissue appears to have no neural function (Stevenson & Wilson, 1974). We have, therefore, examined choline (Ch) uptake in the cornea to compare this property with that of other tissues with a high ACh synthesizing capacity.

The cornea, along with a few mm of attached sclera, was isolated from the rabbit eye. The endothelium was removed. The stromal layer with the intact epithelium was then incubated at 37°C in a holder which permitted both surfaces of the cornea to be bathed in separate media. Uptake of [$^3$H]-Ch (0.03–0.25 μCi contained in 250 μl of Krebs solution) was estimated by removing the epithelium at the end of the incubation and homogenizing in formic acid-acetone (15:85). Radioactivity in an aliquot of homogenate was counted using toluene-Triton-X100 scintillant. Ch and ACh in the remaining homogenate were separated by thin layer chromatography.

On application of Krebs containing [$^3$H]-Ch to the stromal side only, [$^3$H]-Ch appeared in the epithelium within 5 minutes. After 1 h, the radioactivity in the epithelium was ten times that remaining in the medium bathing the stromal side. On the other hand, bathing the epithelium in [$^3$H]-Ch resulted in no accumulation.

In subsequent experiments the uptake of [$^3$H]-Ch from the medium bathing the stromal side was studied over the concentration range 0.1–100 μM. Results were corrected for uptake at 0°C, which was taken to represent passive diffusion of [$^3$H]-Ch into the tissue. The data suggest the existence of a single uptake process showing Michaelis–Menten kinetics (apparent $K_m = 480$ μM and $V_{max} = 15$ nmol/mg protein h$^{-1}$). Uptake of [$^3$H]-Ch appeared to be unaffected by incubation with Na$^+$-free Krebs solution made isotonic with sucrose, although release of free Na$^+$ from stromal bound Na$^+$ could have obscured a low degree of Na$^+$ dependence. Uptake decreased by >50% in the presence of 1 mM dinitrophenol. Inclusion of 100 μM hemicholinium-3 (HC-3) in the incubation fluid caused only 50% inhibition of [$^3$H]-Ch uptake into the epithelium. The proportion of [$^3$H]-Ch recovered from the epithelium as [$^3$H]-ACh was 54 ± 11% (mean ± s.e. mean, $n = 14$) when the incubation was in 1 μM Ch, whereas at 100 μM, only 24 ± 4% ($n = 9$) was converted to [$^3$H]-ACh.

Ch uptake into the epithelium is similar to that found in the human placenta where an uptake with an apparent $K_m$ of 364 μM is associated with 60% conversion of Ch to ACh after 20 min incubation (Welsch, 1976). Despite the large capacity of the epithelium for synthesizing ACh, the cornea appears to lack the high affinity choline uptake exhibited by cholinergic nerves (Yamamura & Snyder, 1973), which is characterized by Na$^+$ dependence and inhibition by low concentrations of HC-3.

This study was supported by grants from the Wellcome Foundation and the Wood Boyd Fund of Glasgow University. S.J.P. is an MRC Scholar.

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## Antagonist affinity constants for adrenomedullary muscarinic receptors

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Muscarinic agonists have been shown to stimulate catecholamine release from the adrenal medulla (Critchley, Tibenham, Ungar, Waite & West, 1975). The action of these secretagogues is inhibited by muscarinic antagonists such as atropine. In view of the high concentrations of antagonist required to block the response, it was decided to investigate the affinity constants of a number of antagonists for the adrenal medullary receptors.

Canine adrenal glands were excised and perfused retrogradely with oxygenated Locke's solution at 37°C through the adrenolumbar vein at a constant

**Table 1** A comparison of the antagonist affinity constants for the adrenal medulla and those values reported for the guinea pig ileum

| *Antagonist* | *Mean dose ratio* | *Adrenomedullary affinity constant ($K_B$ medulla)* | *Reported ileum affinity constant ($K_B$ ileum)* | $\frac{K_B \text{ ileum}}{K_B \text{ medulla}}$ |
|---|---|---|---|---|
| Atropine sulphate,<br>$10^{-6}$ m, $n=3$ | 7.36<br>(6.99–7.80) | $6.36 \times 10^6$ | $10^{9}$* | 157 |
| Oxyphenonium bromide,<br>$2 \times 10^{-7}$ m, $n=3$ | 18.38<br>(9.17–34.20) | $8.69 \times 10^7$ | $5.98 \times 10^9$* | 69 |
| Oxyphenonium bromide,<br>$2 \times 10^{\cdot 2}$ m, $n=3$ | 77.06<br>(54.7–116.6) | $3.80 \times 10^7$ | $5.98 \times 10^9$* | 157 |
| Methylhyoscinium iodide,<br>$2 \times 10^{-7}$ m, $n=3$ | 10.42<br>(5.18–17.69) | $4.71 \times 10^7$ | $5.03 \times 10^9$† | 107 |

* Abramson, Barlow, Mustafa & Stephenson, 1969.
† Barlow, Franks & Pearson, 1973.

flow of 2 ml/minute. The effluent was collected for 30 s periods and the catecholamine content assayed by the trihydroxyindole method (Vendsalü, 1960). The glands were stimulated by two low and two high doses of the specific muscarinic agonist acetyl beta methylcholine ($10^{-8} \rightarrow 10^{-6}$ M) prior to a 90 min equilibration with perfusate containing the antagonist. The response to the agonist was defined as the increment of catecholamine output expressed as a percentage of the resting output. Further higher doses of agonist, in the presence of antagonist, were administered to produce similar responses to those previously obtained enabling the affinity constant of the antagonist to be calculated from the Gaddum/Schild equation (Table 1).

We conclude that in the canine adrenal medulla there exists a population of muscarinic receptors for which the muscarinic antagonists tested showed an affinity two orders lower than that reported for the guinea-pig ileum receptors and this may prove to be a true case of muscarinic receptor heterogeneity.

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## Muscarinic agonists and sympathetic ganglia

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Muscarinic agonists can depolarize sympathetic ganglion cells (see Volle, 1966) but quantitative measurements of their potencies are sparse. We have made some potency measurements on the isolated desheathed rat superior cervical ganglion, superfused with Krebs solution at 25°C bubbled with 95% oxygen/5% carbon dioxide (Brown & Marsh, 1975).

Muscarinic agonists produced a delayed, sustained depolarization of maximum amplitude 0.5–1 mV with extracellular recording (about 20% of a maximal 'nicotinic' depolarization). Comparable responses to carbachol or bethanechol were obtained after suppressing nicotinic depolarization with 2.5 mM hexamethonium. (Hexamethonium reduced the effect

**Table 1** Potencies of muscarinic agonists as depolarizing agents on isolated rat ganglia

| *Agonist* | [1] *$EC_{50}$ (μM)* | [2] *Hyoscine $K_I$ (nM)* |
|---|---|---|
| DL-Muscarine | 0.35 | 0.49 |
| Methylfurmethide | 0.11 | 0.39 |
| Furmethide | 1.9 | 0.54 |
| Pilocarpine | 6.4 | 0.74 |
| [3] AHR-602 | 11 | 0.15 |
| DL-Muscarine* | 2.6 | 5.3 |
| Bethanechol* | 68 | 7.4 |
| Methacholine* | 94 | 2.9 |

[1] Estimated from mean cumulative concentration—response curves from at least 3 experiments.
[2] Estimated from cumulative concentration—response curves for agonist in the presence of 3 nM hyoscine.
[3] *N*-benzyl-3-pyrrolidyl acetate methobromide (Franko, Ward & Alphin, 1963).
* Measured in the presence of 2.5 mM hexamethonium.

of DL-muscarine ten-fold). $EC_{50}$ values for the agonists used lay within the range 0.1–10 μM (Table 1).

Hyoscine inhibited all agonists tested, with an apparent dissociation constant ($K_I$) around 0.5 nM (5 nM in hexamethonium solution). $K_I$ for atropine and lachesine against DL-muscarine were 0.19 and 0.12 nM respectively.

Fair comparability—but not identity—with smooth muscle receptors is apparent.

S.F. is supported by a grant from the University of Azarabadeghan, Iran. We thank A.H. Robins for the gift of AHR-602.

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# A conformational study of enkephalin

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The conformations of peptides can be described (Ramachandran, Ramakrishnan & Sasisekharan, 1963) by means of the torsion angles $\phi$ and $\psi$ about the N—C and C—C bonds in the peptide chain, the torsion angle of the peptide bond being constrained by the planarity of the peptide group. In Figure 1 are shown $\phi$, $\psi$-maps for glycine and alanine. The maps for other amino acids resemble that for alanine, though some amino acids are conformationally more restricted.

The maps can be used to test the configurational specificities of various conformations. For example, the type II $\beta$-bend (Venkatachalam, 1968) contains two residues and the conformations for each of these are shown in Figure 1. Each residue is plotted according to it being glycine, an L-amino acid or a D-amino acid. A D-residue is plotted on the map for an L-residue by inverting the $\phi$ and $\psi$ angles before plotting. It is seen that a D-residue at position 1 and an L-residue at position 2 are disallowed.

Several possible conformations for the pentapeptide leucine-enkephalin have been examined and the configurational specificities predicted for each conformation using $\phi$, $\psi$-maps have been compared with those observed from activity and receptor-binding measurements on leucine enkephalin and analogues containing D-amino acids. Thereby the extent to which the biological data may be explained in terms of the peptide conformation at the receptor has been examined.

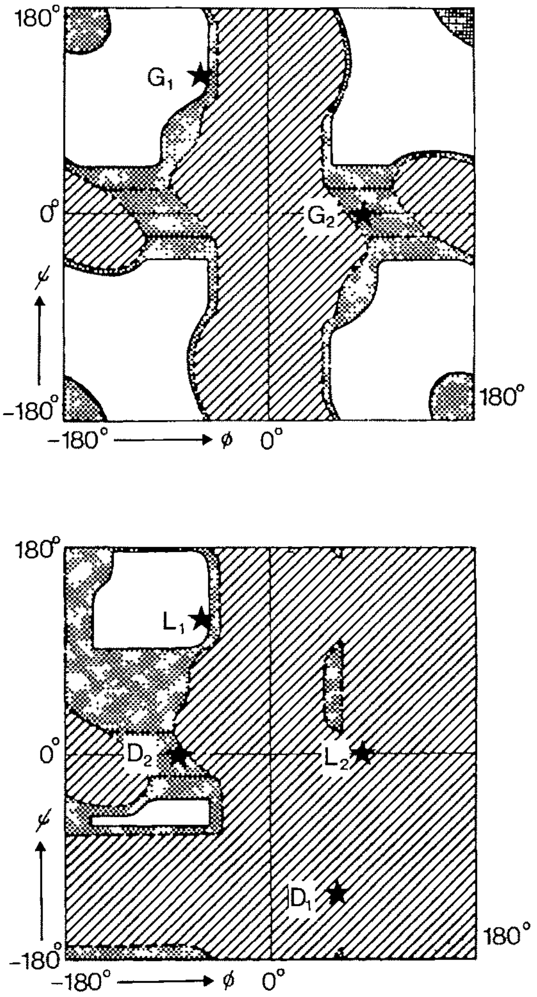

**Figure 1** Conformational maps for glycine (top) and L-alanine. Disallowed combinations of $\phi$ and $\psi$ are hatched. For these, atoms would be too close. Allowed areas are clear. Shaded areas (allowed) correspond to marginally close approaches (e.g. hydrogen bonds). The two residues (1,2) in a type II $\beta$-bend conformation are shown, each plotted as glycine (G), an L-amino acid (L) or a D-amino acid (D).

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# Muscarinic receptors: ionic perturbation of the binding properties

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Both in brain synaptosome preparations and in intact smooth muscle, the binding pattern of antagonists to the muscarinic receptor differs sharply from that of agonists. Whereas the former pattern is described adequately by a simple mass action isotherm, the latter exhibits a functional heterogeneity characterized by flat binding curves exhibiting minimum Hill slopes as low as 0.3. To a first approximation, this pattern can be analysed on the basis of a model (Birdsall & Hulme, 1976) in which two classes of non-interconverting binding sites exist within the receptor population. In both cases, the binding of agonists and antagonists is competitive and mutually exclusive (Birdsall, Burgen, Hiley & Hulme, 1976; Hulme, Burgen & Birdsall, 1976). In the present investigation, it has been possible to perturb the affinity of both agonists and antagonists for brain synaptosome preparations by altering the ionic composition of the medium.

The cerebral cortex from male Wistar rats was used to prepare a $P_2$ pellet containing approximately 1.4 nmol of muscarinic receptor per gram of protein. The pellet was resuspended in appropriate media and binding studies were carried out at 2°C using equilibrium dialysis, a centrifugation assay, or rapid filtration through glass fibre filters. Antagonist binding was measured directly with N-[$^3$H]-methyl-atropine (2.2 Ci/mmol), N-[$^3$H]-methyl-scopolamine (3.3 Ci/mmol), or N-[$^3$H]-propyl-benzilylcholine (40 Ci/mmol).

The binding of antagonists to the receptor is sensitive to the ionic strength. For (–)-N-[$^3$H]-methyl-scopolamine, the value of $pK_d$ decreases from 10.88 in isotonic sucrose-sodium phosphate (10 mM, pH 7) to 9.94 in Krebs–Henseleit solution and to approximately 9.6 in isotonic sucrose-sodium (10 mM, pH 7) containing 1M NaCl. The effects of NaCl, KCl, and $CaCl_2$ are quantitatively similar when compared as a function of ionic strength. In contrast, the trivalent cation $La^{3+}$ causes a greater reduction in $pK_d$ at low ionic strength than do either the monovalent or the divalent ions. With antagonists, neither the shape nor the maximum amplitude of the binding curve is perturbed.

When measured in Krebs–Henseleit solution by the competitive displacement of N-[$^3$H]-propyl-benzilylcholine, the binding curve for the agonist carbachol can be resolved into a high ($pK_d = 7.1$) and a low ($pK_d = 4.5$) affinity component as reported previously (Birdsall *et al.*, 1976; Hulme *et al.*, 1976). As with antagonists, the agonist binding curve is shifted to lower ligand concentrations at low ionic strength. In addition, however, there is a concomitant steepening of the curve. This is caused by the change in the apparent $pK_d$ at the low affinity site ($\Delta pK_d = 1.2$) exceeding that at the high affinity site ($\Delta pK_d = 0.6$). The relative proportion of the two sites appears to remain unchanged. The failure to observe changes in this ratio argues against a facile interconvertibility between the two receptor populations.

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# Muscarinic acetylcholine receptors and cyclic GMP in rat brain

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Slices of rat corpus striatum (250 μm x 250 μm) were incubated as described previously (Minneman & Iversen, 1976), and cyclic (c) GMP was measured in boiled tissue extracts by a radioimmunoassay procedure (Radiochemical Centre, Amersham). In parallel experiments the binding of [$^3$H]-quinuclidinyl benzilate (QNB) (1.4 nM) was measured, using the same slice preparation incubated for 30 min at 20°C. Specific binding of [$^3$H]-QNB was defined as that portion which could be displaced by 1 μM atropine (70–80% of total binding) (Yamamura & Snyder, 1974).

The agonists oxotremorine, arecoline and carbachol caused increases in tissue cGMP with similar time courses and maximum effects, amounting to an approximate doubling over resting cGMP levels (0.7 pmol/mg protein). The cGMP increase reached a maximum after 2 min and declined rapidly thereafter. The cGMP response to oxotremorine (100 μM) was not blocked by α-bungarotoxin or by hexamethonium

**Table 1** Comparison of drug effects on cGMP response and [$^3$H]-QNB binding

| *Agonist* | *$EC_{50}$–μM for cGMP increase* | *Ki for inhibition $^3$H-QNB binding—μM* |
|---|---|---|
| Oxotremorine | 5.6 | 6.4 |
| Arecoline | 22.0 | 61.0 |
| Carbachol | 120.0 | 115.0 |
| *Antagonist* | *$IC_{50}$–nM against cGMP (100 μM oxotremorine)* | *Ki for $^3$H-QNB binding–nM* |
| Scopolamine | 5.6 | 5.8 |
| QNB | 23.0 | 5.0 |
| Atropine | 54.0 | 7.7 |
| Clozapine | 600.0 | 350.0 |
| Thioridazine | 1300.0 | 940.0 |
| Chlorpromazine | 9300.0 | 7700.0 |

(10 μM), but was completely prevented by atropine (1 μM) or QNB (0.1 μM). The muscarinic response was abolished if calcium ions were omitted from the incubation medium, and was additive to the increase in cGMP elicited by sodium azide.

The agonists oxotremorine, arecoline and carbachol yielded $EC_{50}$ values for increasing GMP which agreed well with the corresponding values for these compounds in displacing [$^3$H]-QNB binding (Table 1). The cGMP response thus appears to correspond directly to muscarinic receptor occupancy.

The muscarinic antagonists QNB, scopolamine and atropine and the neuroleptic drugs thioridazine, clozapine and chlorpromazine inhibited the cyclic GMP response to 100 μM oxotremorine. The potencies of the various antagonists in the cGMP and [$^3$H]-QNB tests showed a less close agreement than seen for agonists, and calculated apparent Ka values suggested that the antagonists were more potent in blocking the cGMP response than in displacing [$^3$H]-QNB.

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## Hyperpolarization of myenteric neurones by enkephalin

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In the guinea-pig myenteric plexus, morphine and the enkephalins inhibit the neuronal firing which is induced by a glass suction electrode (Dingledine & Goldstein, 1976; North & Williams, 1976). The inhibitory action of morphine in the myenteric plexus has been attributed to a membrane hyperpolarization (Dingledine & Goldstein, 1976; North & Tonini, 1976). The aim of the present experiments was to determine the effects of the enkephalins upon the membrane properties of myenteric neurones.

Intracellular recordings were made from myenteric ganglia which were removed from the ilea of adult guinea-pigs. The isolated ganglia were immobilized and perfused as previously described (Nishi & North, 1973). Cells were impaled under visual control (Nomarski optics, × 500) with glass micro-electrodes which contained 2M potassium chloride. Drugs were applied by changing the perfusing solution to one which differed only in its content of the drug(s). The majority of Type 1 cells were hyperpolarized by met-enkephalin (100–300 nM). The hyperpolarization began as soon as the met-enkephalin reached the tissue and continued throughout the period of exposure (up to 5 min) and rapidly reversed when the tissue was washed with drug-free Krebs solution. The hyperpolarization ranged in amplitude from 3 to 40 mV, the larger effects being more often seen in cells with low resting membrane potentials. In most cells the hyperpolarization was associated with a fall in membrane resistance—this was evident as a reduction in the amplitude of the membrane potential displacement caused by passing a hyperpolarizing current pulse through the recording electrode by means of a bridge circuit. Cells which responded to met-enkephalin also responded to normorphine (300 nM–1 μM); cells which were unaffected by met-enkephalin were also unaffected by normorphine. Leu-enkephalin had qualitatively similar effects but appeared to be less potent. The hyperpolarization produced by met-enkephalin was reversed by changing to a solution which contained both met-enkephalin and naloxone (20–50 nM); exposure to naloxone alone did not affect the membrane potential but it prevented the action of enkephalin.

Type 2 cells seldom showed any effects of met-enkephalin. A few cells showed a small hyperpolarization (2–5 mV) which passed off during the continued presence of the met-enkephalin.

The functional significance of these effects in the myenteric plexus is not clear. Although the myenteric plexus contains enkephalins (Elde, Hökfelt, Johansson & Terenius, 1976; Smith, Hughes & Kosterlitz, 1976) inhibitory synaptic potentials have not been observed in this tissue. On the other hand, the hyperpolarization provides a basis for the inhibition of spike firing by enkephalins.

This work was supported by US PHS Grant NS 06672.

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## Intracellular recording of the effects of 5-hydroxytryptamine on rabbit superior cervical ganglion cells

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The complex actions of 5-hydroxytryptamine (5-HT) at sympathetic ganglia, which include facilitation and depression of transmission and ganglion cell depolarization (see Wallis & Woodward, 1974), are difficult to elucidate by examining the response of a population of cells. In the work reported here, intracellular recordings were made from rabbit superior cervical ganglia. Rabbits were killed by air embolism and the excised ganglia superfused with Krebs solution at 37°C. Microelectrodes were filled with 2M KCl.

Superfusion of the whole ganglion with 5-HT ($10^{-6}$–$10^{-4}$M) produced only small or negligible changes in resting membrane potential and membrane resistance. In 24 tests on 20 cells, 5-HT had no apparent effect on nine occasions, caused a small depolarization ($<5$ mV) on eleven occasions and a small hyperpolarization on four occasions. The first application of 5-HT more often produced effects than subsequent ones, suggesting the variability in responsiveness might be related to the rapid tachyphylaxis to 5-HT previously reported for these cells (Wallis & Woodward, 1975).

On the other hand, iontophoresis of 5-HT on to the ganglion cell membrane induced substantial depolarizations with a fall in membrane resistance in 29 of 41 cells tested. The iontophoresis electrode was filled with 20 mM 5-HT creatinine sulphate and positioned near an impaled cell, using a separate micromanipulator. Some cells responded to ejection currents of 20 nA for 100 ms, others only after passing greater currents for several seconds. Only outward current evoked depolarizations. Twelve cells were apparently unresponsive to 5-HT. Lack of response was not easy to establish unequivocally because of difficulty in visualizing the position of the iontophoresis electrode with respect to the impaled cell.

Iontophoresis of 5-HT induced depolarizations which were graded in amplitude (up to 19 mV) as the duration or the intensity of the ejection current was altered. The time to peak of the depolarization was typically 1–3 seconds. Membrane resistance fell by 15–55% at the peak of the depolarization. An after-hyperpolarization sometimes followed the depolarization by which time membrane resistance had returned to control values. The depolarization was sometimes clearly divided into an initial and a late depolarization, evident as a hump on the falling phase of the initial response. The 5-HT depolarization did not decline in amplitude when elicited at frequencies of 0.033 Hz; at 0.1 Hz the amplitude of second and subsequent responses declined by 25–30%. The amplitude of the 5-HT depolarization was augmented by hyperpolarization and decreased by depolarization of the soma membrane of the ganglion cell.

Iontophoretic currents were not exciting nerve terminals and releasing acetylcholine because (a) iontophoretic responses were unaffected by hexamethonium ($5 \times 10^{-4}$ M), whereas synaptic potentials were depressed by 70%; (b) responses were reduced in amplitude by cyproheptadine ($5 \times 10^{-5}$ M) by about 50%; (c) responses were reduced in amplitude by superfusing the ganglion with $10^{-6}$ M 5-HT and nearly abolished on superfusing with $5 \times 10^{-5}$ M 5-HT, although these concentrations of 5-HT did not affect the synaptic potentials.

Supported by US PHS Grant NS 06672 and by a Wellcome Research Travel Grant to D.I.W.

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## The uptake of $^{45}$calcium into slices of rat cerebral cortex: effect of depolarizing stimuli

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Previous studies have shown that neurotransmitter uptake is markedly dependent on slice size (Levi & Raiteri, 1973) and it has been postulated that varying slice size can be used to examine uptake into neuronal and glial cells (Riddall, Leach & Davison, 1976; Leach, Riddall & Winkley, 1976). The intracellular concentration of calcium may be increased by various depolarizing stimuli and the effects of these stimuli on $^{45}$calcium uptake into slices of rat cerebral cortex has been examined.

Two sizes of slice were employed (0.1 × 0.1 × 2 mm and 0.4 × 0.4 × 2 mm) and they were prepared in the manner described by Iversen & Neal (1968) except that pH 7.4 Tyrode medium was used. Slices were preincubated at 37°C for 15 min and portions (equivalent to 20 mg wet wt.) of this suspension were transferred to Tyrode (containing the depolarizing stimuli) or to potassium stimulation media both of which contained $^{45}Ca^{2+}$ (0.1 µCi ml, 1.78 mM). Flasks were incubated for varying times and uptake was terminated by the addition of 1 ml of quench medium (24 mM EGTA-tris, 72 µM ruthenium red in Tyrode). The suspension was immediately filtered under vacuum, washed, and the resulting tissue sample was digested and counted by the method of Dent & Johnson (1974).

The basal flux of $^{45}Ca^{2+}$, i.e. 'exchange', was greater in 0.4 mm slices than in 0.1 mm slices giving asymptotic 'uptake' values of $5.64 \pm 0.26$ and $1.94 \pm 0.11$ µmol $Ca^{2+}$/g wet wt. ($P < 0.01$) respectively. In the presence of ouabain (0.1 mM) the corresponding asymptotic values for $^{45}Ca^{2+}$ uptake into 0.4 mm and 0.1 mm slices were increased to $12.27 \pm 0.38$ and $3.44 \pm 0.23$ µmol $Ca^{2+}$/g wet wt. ($P < 0.01$). Pre-treatment with the calcium ionophore, A23187 (200 µg/ml) increased $^{45}Ca^{2+}$ uptake into both slice sizes at 10 and 60 min of incubation. Glutamate (5 mM) had no effect on $^{45}Ca^{2+}$ uptake into either slice size.

Potassium exhibited different effects on the uptake of $^{45}Ca^{2+}$ into the two slice sizes. $K^+$ (49 mM) stimulation of $^{45}Ca^{2+}$ uptake into 0.1 mm slices was characterized by a rapid initial phase which lasted for approximately one minute. This rapid initial phase was followed by a slower phase which was similar in magnitude to the uptake found in the absence of elevated $K^+$. In contrast $K^+$ (49 mM) had no significant effect on $^{45}Ca^{2+}$ uptake into 0.4 mm slices.

Uptake mechanisms for putative neurotransmitters into small slices (0.1 × 0.1 × 2 mm) of rat cerebral cortex are well documented. Conversely, there are little, if any, data relating to the release mechanisms operating in these slices. The results presented above indicate a prominent role for glial cells in the regulation of $Ca^{2+}$ flux. This role is demonstrated by the effects of slice size and depolarizing stimuli on $^{45}Ca^{2+}$ uptake into rat cerebral cortex.

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## Prolongation of post-synaptic inhibition by barbiturates

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When slices of guinea-pig olfactory cortex are incubated *in vitro* and the lateral olfactory tract (LOT) is stimulated the evoked excitatory post-synaptic potential (EPSP) recorded within the mono-synaptically-innervated neurones is followed by an inhibitory post-synaptic potential (IPSP). This IPSP is manifest by a large conductance increase and a small depolarization at the normally-recorded resting membrane potential of −76 mV (Scholfield, 1976). The present report concerns a unique and substantial prolongation of this IPSP by barbiturates.

Surface slices of guinea-pig olfactory cortex were maintained in a superfusing stream of Krebs solution at 25°C bubbled with 95% oxygen/5% carbon dioxide, and neurones in the prepyriform cortex were impaled with glass microelectrodes filled with K acetate as described previously (Scholfield, 1976). Extracellular surface potentials were monitored with a saline-filled microelectrode. The LOT input was stimulated at $<1$ min intervals.

Sodium pentobarbitone (100 μM) increased the time to half-decay of the post-synaptic inhibitory conductance from $0.17 \pm 0.04$ to $1.68 \pm 0.22$ s (mean ± s.e. mean, 5 slices). The apparent reversal potential for the IPSP (initially −70 to −69 mV) shifted some 10 mV in the depolarizing direction during the course of the individual barbiturate-prolonged IPSP. Spikes were not generated during this prolonged depolarization.

The initial EPSP, spike potential and resting input resistance and time constant were unchanged below 200 μM pentobarbitone. At higher concentrations input resistance fell substantially, the membrane depolarized towards −60 mV and all synaptic potentials were depressed.

During a train of LOT stimuli the second EPSP is shunted by the IPSP and fails to generate a spike, but subsequently recovery normally ensues such that repetitive EPSP-spike combinations result (Scholfield, 1976). In contrast, pentobarbitone not only prevented recovery but intensified EPSP depression during repetitive LOT stimulation. This accords with previous observations made with extracellular electrodes (Scholfield & Harvey, 1975).

Effects of pentobarbitone within the range 20 μM–1 mM could be replicated by phenobarbitone (0.2–5 mM). In contrast procaine (2–10 mM) simply attenuated the spike and depressed all post-synaptic potentials. This confirms the previously-drawn (Scholfield & Harvey, 1975) distinction between the actions of barbiturates and local anaesthetics on synaptic processes in this preparation.

The present results accord with observations in other parts of the CNS *in vivo,* such as those of Nicholl, Eccles, Oshima & Rubia (1975). Since selective potentiation of the IPSP occurs at concentrations within or below the range expected during anaesthesia, such an effect may contribute to the actions of barbiturates *in vivo.*

This study was supported by a grant from the Medical Research Council.

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# Non-additive anaesthetic effects of alphaxalone and methohexitone

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In 1937 Meyer suggested that the correlation between the anaesthetic potency of a substance and its lipid solubility could be summarized as follows: anaesthesia commences when any chemically indifferent substance has attained a certain molar concentration in the lipids of the cell; this concentration depends on the species but is independent of the nature of the anaesthetic. Subsequently Mullins (1954) proposed that anaesthesia commences when a certain critical volume fraction of anaesthetic has been achieved in the cell membranes. Both hypotheses predict that the effects of combinations of anaesthetics should be additive. This has been verified with mixtures of gaseous and volatile anaesthetics (Miller, Wahrenbrock, Schroeder, Knipstein, Eger & Buechel, 1969). To test this prediction for non-volatile anaesthetics we chose to investigate the anaesthetic potencies of mixtures of short-acting intravenous anaesthetics.

Male Wistar rats (150–200 g) received, into the tail vein, injections of methohexitone, alphaxalone or alphadalone acetate either alone or as mixtures of two compounds. The duration of the loss of righting reflex was recorded. Each rat received only one injection.

Rats injected with either alphaxalone (3 mg/kg), alphadalone acetate (12 mg/kg) or methohexitone (12 mg/kg) lost their righting reflex for about 8 minutes. Rats injected with a mixture of alphaxalone (1.5 mg/kg) and alphadalone acetate (6 mg/kg) also lost their righting reflex for about 8 min as predicted by the theories. However, rats injected with a mixture of alphaxalone (1.5 mg/kg) and methohexitone (6 mg/kg) lost their righting reflex for about 11 min (see Fig. 1).

These behavioural results are supported by unpublished electrophysiological observations with similar mixtures which have shown that alphaxalone potentiates the depressant action of methohexitone and other barbiturates on synaptic transmission in isolated preparations of olfactory cortex. Therefore the potentiation between methohexitone and alphaxalone presumably reflects a specific drug interaction within the central nervous system. These results are not compatible with either the simple lipid solubility hypothesis of Meyer or with the critical volume hypothesis of Mullins.

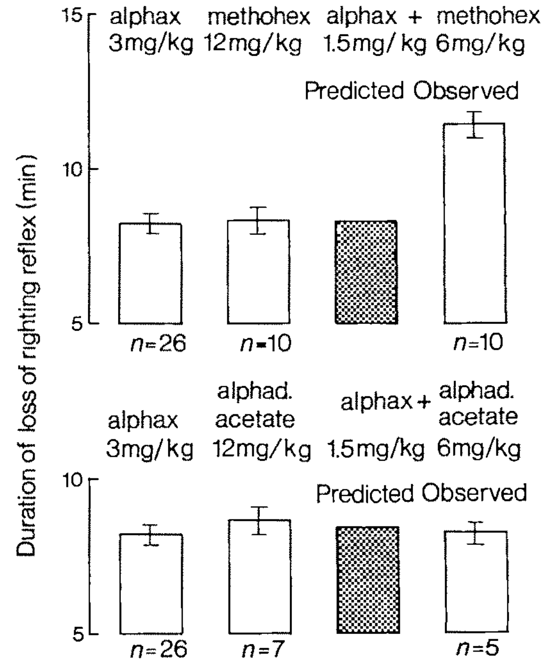

**Figure 1** The height of each column represents the mean duration of the loss of righting reflex (±s.e. mean) produced by i.v. injection of anaesthetic or combination of anaesthetics shown above. The duration of the loss of righting reflex caused by a mixture of alphaxalone and methohexitone was significantly longer than that produced by either anaesthetic given alone ($P < 0.001$ by 2 tailed t-test).

We wish to thank Dr B. Davis of Glaxo Research Ltd. for the steroids.

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# Stress-induced partial insomnia: a model for the evaluation of potential hypnotic compounds

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The testing of new hypnotics has usually been performed in normal animals (Babbini, Torrielli, Strumia, Gaiardi, Bartoletti & De Marchi, 1975). Logically, better assessment of efficacy would be to screen the drugs in insomniac animals. There are several published methods for the selective deprivation of different sleep states (Albert, Cicala & Siegal, 1970; Morden, Mitchell & Dement, 1967; Ogilvie & Broughton, 1976) but much of this work may be unsuitable for evaluation of drug activity for example, in the 'island technique' there is no facility for quantitating the reversal of the insomnia. This paper will describe a technique of inducing reversible insomnia which is readily and accurately quantitated and can be used for evaluation of hypnotic properties. Two benzodiazepines, namely RU 31158 [6-(ortho-chlorophenyl)-1,2-dihydro-2(N-methyl-piperazin-1-yl)-methylene-8-nitro-1H,4H-imidazo [1,2-a][1,4]benzodiazepin-1-one methanesulphonate] (Taylor, 1976) and flunitrazepam, as well as phenobarbitone, morphine and chlorpromazine have been examined in this test.

Male Wistar CFHB rats (200–275 g) were used in the study in which the electrocorticogram (ECoG) and electromyogram (EMG) were automatically analysed to quantitate the sleep states (Johns, Piper, James, Birtley & Fischer, 1976). Following a 40 h acclimatisation period, an 8 h placebo treated control recording was performed. On the succeeding day, placebo or drug was given orally and the animals were stressed. The stress was induced by foot electroshock delivered through the EMG recording leads and the metal grid floor, 0.5 mA for 30 s every 30 min, 15 msec pulse width at 1 Hz. During the electroshock period the ECoG and EMG recording circuits were automatically interrupted by a two-way relay switch system. The results of the stress procedure on 10 animals was to significantly ($P \leqslant 0.05$) increase the arousal and slow wave sleep (SWS) I phases of the sleep cycle. There was a significant ($P \leqslant 0.05$) decrease in SWS II and paradoxical sleep throughout the eight hours.

RU 31158 (8 mg/kg, $n = 10$) and flunitrazepam (4 mg/kg, $n = 10$) were effective sleep inducing agents in this model. Both compounds restored the arousal and paradoxical sleep patterns to a control profile and partially restored the SWS II pattern, although RU 31158 was most active for the first 3 h and flunitrazepam reached peak activity on SWS II at four hours.

Phenobarbitone (50 mg/kg, $n = 10$) was active in suppressing the stress increased arousal. However, there was a significant ($P \leqslant 0.05$) increase in SWS I and a concomitant decrease in SWS II. Paradoxical sleep was suppressed. Chlorpromazine (8 mg/kg, $n = 10$) was ineffective as a sleep inducing compound. There was a significant increase only in SWS I at 2–4 h which reflects the action of chlorpromazine on normal animal EEG (Babbini, Gaiardi & Bartoletti, 1975). Morphine (17.2 mg/kg, $n = 10$) was ineffective in this model.

The results suggest that the stress-induced partial insomnia in the rat is a reliable model for screening potential hypnotic compounds. The two benzodiazepines, RU 31158 and flunitrazepam presented good hypnotic profiles whilst representative drugs from other major CNS categories were either only partially active or completely inactive.

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# Pre-synaptic $\alpha$-adrenoceptor regulation of the twitch response of the mouse vas deferens

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Noradrenaline inhibits the twitch response of the mouse vas deferens and this inhibition is blocked by phentolamine (Jenkins, Marshall & Nasmyth, 1976). The mechanism of this inhibition has now been investigated using drugs that selectively affect pre- and post-junctional $\alpha$-adrenoceptors.

The twitch response to electrical stimulation of the mouse vas deferens (256 mA at either 0.2 Hz, 2 ms, or 0.1 Hz, 1 ms) was inhibited by noradrenaline (0.06–48 µM) with an $ID_{50}$ of 1.5 µM. Clonidine, a selective pre-synaptic $\alpha$-adrenoceptor agonist (Starke, Endo & Taube, 1975), also inhibited the twitch (0.56–56 nM, $ID_{50}$ 6.25 nM) as did tyramine (3–30 µM), an indirect acting amine (Trendelenburg, 1961), $ID_{50}$ 18 µM.

Inhibiting uptake with cocaine (10 µM) and oestradiol (3.7 µM) increased the noradrenaline inhibition, did not affect clonidine inhibition and abolished the tyramine response.

The $\alpha$-adrenoceptors mediating the inhibition were investigated using the selective pre-junctional antagonist yohimbine (Starke, Borowski & Endo, 1975). The twitch response of the mouse vas deferens (0.25–2.0 ms, 0.2 Hz) was potentiated by yohimbine, 3.2 to 128 nM in a dose-related manner. The inhibition produced by noradrenaline and clonidine was reduced by yohimbine in doses below 10 nM.

Phenylephrine, an agonist selective for post-junctional $\alpha$-adrenoceptors (Starke *et al.*, 1975) potentiates the twitch response and also produces a contraction of the vas deferens. Both effects were abolished by phentolamine (Jenkins *et al.*, 1976). However, neither action of phenylephrine was antagonized by yohimbine (10 nM).

Thymoxamine (0.3 µM) a selective post-junctional $\alpha$-adrenoceptor antagonist (Drew, 1976), inhibited the contraction and potentiation of the twitch produced by phenylephrine (0.15–3.0 µM) but did not affect the inhibition produced by clonidine (3.0–18.0 nM).

These results demonstrate that agonists selective for presynaptic $\alpha$-adrenoceptors produce inhibition of the twitch response of the mouse vas deferens and this is blocked by the selective pre-synaptic antagonist yohimbine. Conversely, phenylephrine, an agonist selective for post-synaptic $\alpha$ receptors, potentiated the twitch response and caused contractions of the vas deferens, effects which were selectively blocked by a post-synaptic $\alpha$-receptor antagonist. The inhibition produced by endogenous noradrenaline released by tyramine also appears to be mediated via pre-junctional $\alpha$-adrenoceptors. This, together with the potentiation of the twitch by yohimbine, suggests that the motor response of the mouse vas deferens to electrical stimulation may be controlled through pre-synaptic $\alpha$-adrenoceptors.

NBS is an MRC scholar.

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## Pre-synaptic $\alpha$-adrenoceptors and the inhibition by uptake blocking agents of the twitch response of the mouse vas deferens

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Field stimulation of the mouse vas deferens releases noradrenaline (Henderson, Hughes & Kosterlitz, 1972; Jenkins, Marshall & Nasmyth, 1975) and elicits a twitch response. Inhibition of neuronal uptake by cocaine potentiates the response of sympathetically innervated organs to electrical stimulation, especially at low rates of stimulation (< 1 Hz) (Iversen, 1973). The effect of uptake inhibitors in the mouse vas deferens has now been investigated.

The inhibition of noradrenaline uptake by cocaine, cocaine plus oestradiol, or phenoxybenzamine was measured in intact vasa deferentia. A single vas deferens was incubated with [7-$^3$H]-(−)-noradrenaline (10 ng/ml; specific activity 9.8 Ci/mmol) for 10 min, washed, homogenized and the catechols then adsorbed onto alumina. The tritium counts were taken to represent [$^3$H]-noradrenaline uptake and this was $28.53 \times 10^5$ d min$^{-1}$ g$^{-1}$ tissue controls. The addition of cocaine (10 µM) decreased this by 93%; cocaine (10 µM) plus oestradiol (3.7 µM) by 87%, and phenoxybenzamine (15 µM) by 89% ($P < 0.001$ for all 3 cases).

The effect of these drugs on the twitch response was examined. An isolated vas deferens was stimulated at 0.2 Hz, 256 mA and pulse widths of 0.25–2.0 ms. The addition of cocaine (10 µM) or cocaine (10 µM) plus oestradiol (3.7 µM) significantly inhibited the twitch at all pulse widths ($P < 0.05$). Conversely, phenoxybenzamine (15 µM) significantly potentiated the twitch at all pulse widths ($P < 0.001$).

The differing effect of these uptake inhibitors on the twitch may be related to the pre-synaptic $\alpha$-adrenoceptor blocking activity of phenoxybenzamine, a property not shared by the other uptake inhibitors. Stimulation of these receptors by noradrenaline can inhibit the twitch response in the mouse vas deferens (Marshall, Nasmyth, Nicholl & Shepperson, this Meeting). To test this possibility yohimbine, a selective pre-synaptic $\alpha$-receptor blocking agent (Starke, Borowski & Endo, 1975) was used. Yohimbine (3.2–128 nM) reversed the inhibition produced by cocaine plus oestradiol and the twitch was now potentiated.

These results suggest that, in the presence of cocaine and oestradiol, noradrenaline released by electrical stimulation of the mouse vas deferens inhibits the twitch response via a pre-synaptic $\alpha$-adrenoceptor.

NBS is an MRC scholar.

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## Further evidence for dopaminoceptors in the vas deferens

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There are conflicting reports about the neurotransmitter role of noradrenaline (NA) in the vas deferens of most species because large doses of NA are required to elicit a contraction (Graham, Al Katib & Spriggs, 1968; Birmingham, 1970). In most studies contractions of the vas deferens are resistant to alpha-adrenoceptor antagonists except at very high concentrations (Ambache & Zar, 1971). These led Ambache & Zar (1971) to challenge the concept that NA acts as the motor neurotransmitter substance in the vas deferens.

However there is little or no study on the effect of dopamine (DA) on the vas deferens. Tayo (1977) reported that in the isolated vas deferens of the rat, DA was more potent than NA whereas the reverse was true in the guinea-pig. Furthermore, pimozide reduced or abolished DA-induced effect without modifying NA-induced contraction. Guanethidine or 6-hydroxy-dopamine induced supersensitivity towards NA whereas there was none towards DA (Tayo, unpublished observations).

The aim of the present studies is to investigate the presence of prejunctional inhibitory DA receptors in the rat vas deferens. The vas deferens was isolated with the hypogastric nerve intact and mounted in a 20-ml organ bath containing Tyrode solution bubbled with 5% $CO_2$ in oxygen at 35°C.

Stimulation of the nerve produced contractions which were readily abolished by guanethidine. DA (5 pg/ml–50 ng/ml) produced graded decreases in the contractions. NA in the same range produced quantitatively less effect. Higher concentrations of DA and NA (500 ng/ml–5 μg/ml) directly stimulated the vas smooth muscle to contract but reduced or totally abolished the effect of nerve stimulation. This effect was more marked at lower than at higher frequencies of stimulation.

DA receptor antagonists, pimozide (5 ng/ml–50 ng/ml) and haloperidol (500 ng/ml–5 μg/ml), increased the effect of nerve stimulation. These agents abolished the inhibitory action of DA on nerve stimulation. The effect of NA was also abolished by haloperidol but not by pimozide.

The regulation of transmitter release by prejunctional receptors have been reported in various tissues (Hotta, 1969; Langer, 1973; Rand, McCullough & Story, 1975; Stjarne, 1975; Hedqvist, 1976). The present results indicate that low concentrations of DA probably act on prejunctional inhibitory dopaminoceptors to modify neurotransmission by reducing the rate of transmitter release. The increase in response produced by DA antagonists is compatible with this concept since blockade of inhibitory receptors would uncover more of the excitatory receptors. The inhibition of nerve stimulation produced at higher DA concentration (prejunctional) and the excitation at the same concentrations (postjunctional) suggest a possible dual effect of DA.

The gift of haloperidol and pimozide by Squibb Inc. and Janssen Pharmaceuticals is gratefully acknowledged.

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# Pharmacological characterization of presynaptic $\alpha$-adrenoceptors which regulate cholinergic activity in the guinea-pig ileum

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Field stimulation of the guinea-pig ileum releases acetylcholine from cholinergic nerves and causes contraction of the longitudinal muscle. The contraction is inhibited by $\alpha$- and $\beta$-adrenoceptor agonists. $\beta$-Adrenoceptor agonists stimulate $\beta$-adrenoceptors located on the smooth muscle, thereby causing muscle relaxation, whereas $\alpha$-adrenoceptor agonists act by reducing the release of acetylcholine from the cholinergic nerves. The $\alpha$-adrenoceptors involved are located on the cholinergic nerve terminals (Knoll & Vizi, 1971; Kosterlitz & Lees, 1972).

The release of noradrenaline from sympathetic nerves is modulated by $\alpha$-adrenoceptors located at the nerve terminals. These presynaptic $\alpha$-adrenoceptors differ from postsynaptic $\alpha$-adrenoceptors in their sensitivity to agonists (Starke, Endo & Taube, 1975; Drew, 1976) and to antagonists (Dubocovich & Langer, 1974; Blakely & Summers, 1976; Drew, 1976). The presynaptic $\alpha$-adrenoceptors are characteristically less sensitive than the postsynaptic $\alpha$-adrenoceptors to the agonist effects of phenylephrine and methoxamine and to the antagonist actions of thymoxamine, labetalol and phenoxybenzamine. Langer (1974) has suggested, therefore, that $\alpha$-adrenoceptors should be subclassified as $\alpha_1$ (postsynaptic type) and $\alpha_2$ (presynaptic type).

The differential sensitivity of pre- and postsynaptic adrenoceptors to $\alpha$-adrenoceptor agonists and antagonists has been used to characterize the $\alpha$-adrenoceptors located on the cholinergic nerve terminals in the guinea-pig ileum. Segments of ileum were suspended under 0.5–1.0 g tension in Krebs solution, at 37°C, containing propranolol (0.3 µg/ml). Field stimulation (0.1 Hz; 1 ms; supramaximal voltage) was delivered using platinum electrodes, 12 mm apart, and the resulting twitch responses were recorded isometrically.

Agonists were added to the bathing fluid in a cumulative-concentration schedule. Clonidine (0.1–10 ng/ml), oxymetazoline (0.1–100 ng/ml) and xylazine (0.1–100 ng/ml) caused concentration dependent reductions in the twitch response. The maximum effects of these drugs corresponded to 85–95% inhibition of the twitch response. Phenylephrine and methoxamine were 1000–10,000 times less potent than clinidine.

Phentolamine (0.01, 0.1 and 1.0 µg/ml) and piperoxan (0.03, 0.1 and 0.3 µg/ml) were potent antagonists of the inhibitory effect of clonidine. In contrast, thymoxamine (1, 3 and 10 µg/ml) was only weakly active and labetalol (0.3 and 1 µg/ml) was inactive against clonidine.

These results suggest that the presynaptic $\alpha$-adrenoceptors located on the parasympathetic nerve terminals in the guinea-pig ileum are of the same type as those located on cholinergic nerve terminals—that is $\alpha_2$.

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## The effects of dopamine and dobutamine on isolated coronary vascular smooth muscle of the pig

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Goldberg (1972) has postulated the presence of specific dopamine receptors in renal and mesenteric vascular smooth muscle. There is also some evidence that such receptors may also play a role in the coronary dilator effects of dopamine (Schuelke, Mark, Schmid & Eckstein, 1971; Toda & Goldberg, 1975). We have examined this possibility by comparing the effects of dopamine, dobutamine, noradrenaline and isoprenaline on pig isolated coronary artery strips bathed in Krebs-Henseleit solution (37°C) bubbled with carbogen.

In strips contracted with submaximal concentration of $K^+$ (10–30 mM) dopamine ($0.2–5 \times 10^{-3}$ M), dobutamine ($0.3–9 \times 10^{-6}$ M) and noradrenaline ($0.5–7.5 \times 10^{-7}$M) caused further dose-dependent increases in vascular tone. After blockade of $\alpha$-adrenoceptors with phenoxybenzamine ($1.6 \times 10^{-5}$ M) these three agonists caused dose-dependent relaxations of the tissue. Isoprenaline ($0.3–5.6 \times 10^{-6}$ M) and the phosphodiesterase inhibitor DL-4-(3 butoxy-4-methoxy benzyl)-2-imidazolidinone (Ro1724) ($0.4–6.2 \times 10^{-7}$M) also caused dose-dependent relaxations. Pretreatment of the tissues with propranolol ($3 \times 10^{-6}$ M) caused parallel shifts to the right of the dose-response curves to isoprenaline and noradrenaline but left the responses to dopamine, dobutamine and Ro1724 unaffected. The relaxant responses to dopamine and dobutamine were also unaffected by pretreatment with either atropine ($4 \times 10^{-6}$ M), apomorphine ($3 \times 10^{-6}$ M) or ergometrine ($2 \times 10^{-6}$ M), were significantly potentiated by haloperidol ($3 \times 10^{-6}$ M) and pimozide ($2 \times 10^{-6}$M) and were abolished by the phosphodiesterase stimulant, imidazole ($1.5 \times 10^{-4}$ M).

In concentrations which did not themselves produce a measurable response both dopamine ($1.3 \times 10^{-4}$ M) and Ro1724 ($0.2 \times 10^{-7}$ M) caused a significant (7-fold) shift to the left of the isoprenaline dose-response curves.

These results suggest that the direct vasodilator actions of dopamine and dobutamine in large coronary vessels in the pig do not involve stimulation of either $\beta$-adrenoceptors or specific dopamine-receptors, but may result from inhibition of cyclic nucleotide phosphodiesterase. Detailed analysis of the time-course of the coronary vasodilator actions of dopamine and dobutamine also suggest an intracellular mechanism rather than interaction with a membrane-bound receptor.

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## Prazosin, a selective antagonist of post-synaptic $\alpha$-adrenoceptors

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Among its pharmacological effects, the antihypertensive drug prazosin can bring about a functional blockade of $\alpha$-adrenoceptors quite unlike the occupancy block caused by conventional $\alpha$-adrenoceptor blocking agents (Constantine, McShane, Scriabine & Hess, 1973). Similarly, unlike conventional $\alpha$-adrenoceptor blocking agents, prazosin did not cause tachycardia or renin release in dogs (Constantine *et al.*, 1973; Massingham & Hayden, 1975).

In light of the realization that at the terminal ramifications of sympathetic C fibres local control mechanisms operate which modulate transmitter output through positive and negative feedback mechanisms, we decided to compare the effects of prazosin with phenoxybenzamine on pre- and post-synaptic $\alpha$-adrenoceptors (Langer, 1974; Starke, Endo & Taube, 1975).

The affinity of prazosin and phenoxybenzamine for pre-synaptic receptors was estimated by measuring

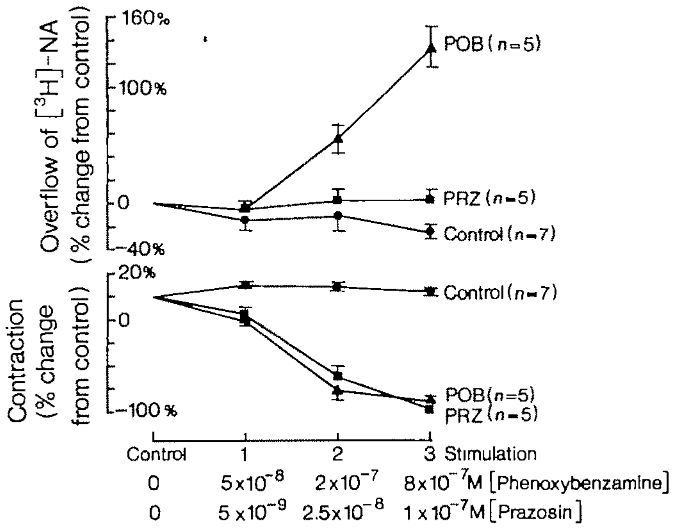

**Figure 1** Effect of prazosin and phenoxybenzamine on contraction and $^3$HNA overflow in rabbit pulmonary artery after blockade of uptake 1 and uptake 2. The control stimulation is the mean of 2 responses.

the stimulation-induced overflow of tritiated noradrenaline, whilst affinity for post-synaptic α-adrenoceptors was estimated by measuring the changes in tension elicited by nerve stimulation in superfused rabbit pulmonary artery preparations as described by Su & Bevan (1970).

The effects of prazosin and phenoxybenzamine were studied both in the absence and presence of desipramine (0.6 μM) and normetanephrine (10 μM) since phenoxybenzamine is known to block both neuronal and extraneuronal uptake of catecholamines (Iversen, 1971). In fact, similar results were obtained both in the presence and absence of uptake inhibitors.

The figure shows that prazosin has a remarkable specificity for post-synaptic adrenoceptors with little or no affinity for pre-synaptic. Prazosin, in concentrations which produce similar antagonism of post-synaptic events, caused virtually no increase in the overflow of tritiated noradrenaline whilst phenoxybenzamine caused a marked increase.

It is concluded that prazosin possesses specificity for post-synaptic α-adrenoceptors and that this provides an explanation for its apparent lack of tachycardia, tolerance and renin release which are observed with conventional α-adrenoceptor blocking agents.

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# The nature of central dopamine receptor supersensitivity

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Receptors within the peripheral nervous system develop the phenomenon of supersensitivity if they are deprived of their natural chemical transmitter for any length of time (Thesleff, 1960; Trendelenburg, 1966). The same principle has been applied to the central nervous system, and, for example, has been suggested to explain the changes observed in animal behaviour following manipulation of the central dopaminergic mechanisms. The pharmacological basis of the rotating rodent is an important application of this principle, where degeneration of one nigro-neostriatal dopaminergic pathway is believed to be associated with the production of supersensitive striatal dopamine receptors. This phenomenon results in an animal that circles away from the side of the lesion when systemically administered directly-acting dopamine agonists, due, it is suggested, to a preferential stimulation of these supersensitive receptors (Ungerstedt, 1971).

In the periphery, 're-innervation' of the denervated receptor is associated with a return of receptor function towards normal (Thesleff, 1960). Since complete functional re-innervation is not possible within the central nervous system, little is known of the nature of the dopamine receptor rendered supersensitive by presynaptic degeneration. However, a partial 're-innervation' of these central receptor sites can be achieved by the chronic oral administration of levodopa (L-DOPA), the direct precursor of dopamine. Such would be its effect in the L-DOPA therapy of Parkinson's disease.

The turning mouse model of von Voigtlander & Moore (1973) was used as a test of the function of supersensitive striatal dopamine receptors. Dose-circling response curves to systemically administered L-DOPA (range 10–200 mg/kg, plus the peripheral decarboxylase inhibitor, carbidopa, 25 mg/kg) were conducted in two groups of mice with unilateral 6-hydroxydopamine induced destruction of striatal dopaminergic nerve terminals. One of these groups was fed L-DOPA chronically in the diet.

Although chronic oral administration of L-DOPA for two months increased spontaneous locomotor activity and induced spontaneous contraversive circling behaviour, it did not significantly alter the circling response induced by systemically administered L-DOPA. Similarly, withdrawal of L-DOPA from the diet did not influence L-DOPA induced circling behaviour, although both spontaneous locomotor activity and spontaneous turning behaviour returned to control levels. Biochemically, chronic administration of L-DOPA to a further group of mice caused only modest (25–30%) increases in cerebral dopamine concentrations on both the lesioned and intact sides; noradrenaline and 5-hydroxytryptamine levels were unaltered.

The behavioural results suggest that central dopaminergic denervation 'supersensitivity' is permanent in nature. However, as in Parkinson's disease, administration of L-DOPA to this animal model did not restore the dopamine concentrations on the side of the lesion to normal. Thus functional re-innervation may have been only partial, and the possibility that complete functional re-innervation could reverse dopamine receptor supersensitivity cannot be dismissed.

These somewhat unexpected results may convey a warning. Perhaps it is not strictly correct to compare animal models, where apparent supersensitivity is developed very rapidly following an acute lesion, with human neurological disorders such as Parkinson's disease, where the condition may take months or years to achieve complete functional loss of presynaptic terminals.

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# DEMONSTRATIONS

## The assay of tissue kallikrein in rat intestine

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Werle (1960) reported the presence of trypsin-activated hypotensive activity, thought to be kallikrein, in the gut wall of rats. He found that a change from normal diet to diets consisting solely of meat, eggs or fat caused increases in this activity in the small intestinal wall. Kallikreins form kinins which have powerful actions on blood flow, vascular permeability and gut motility. A method is described for measuring localized changes in kinin-forming activity in intestinal tissue.

A kinin-forming substrate (KFS) was prepared from human plasma by a modification (Zeitlin, Singh, Lembeck & Theiler, 1976) of the method of Amundsen, Nustad & Waaler (1963). Fasted male Wistar rats were killed, exsanguinated and the gut freed of blood as described by Zeitlin *et al.* (1976).

The stomach, duodenum, jejunum, terminal ileum, caecum, proximal colon and distal colon were removed, opened, washed in Krebs solution and homogenized separately in 0.1 N-HCl (10 ml per g wet weight). Homogenates were allowed to stand for 15 min to destroy kininase, neutralized using 2 N-NaOH and activated autolytically (Zeitlin, 1971). Homogenates were centrifuged and aliquots of supernatant were incubated with excess KFS in 0.1 M-glycine buffer (pH 8.5, 37°C). Enzymic action was stopped by boiling and bradykinin-like activity was assayed using contractions of isolated superfused oestrous rat uterus. The gut extracts contained negligible kininase. Formation of bradykinin-like activity was linear with time and enzyme concentration until 80% substrate consumption. Bradykinin-like activity was characterized by parallel bio-assay.

In normally fed rats, all gut tissues contained kallikrein (Figure 1) with least in the stomach (0.02 µg bradykinin equiv. min$^{-1}$ g$^{-1}$ tissue) and a single large peak in the caecum (0.5 µg min$^{-1}$ g$^{-1}$). In 24 h fasted rats allowed water *ad libitum*, the level in the duodenum more than doubled to 0.14 µg min$^{-1}$ g$^{-1}$ ($P < 0.05$) while that in the caecum fell to less than half of the fed value ($P < 0.05$).

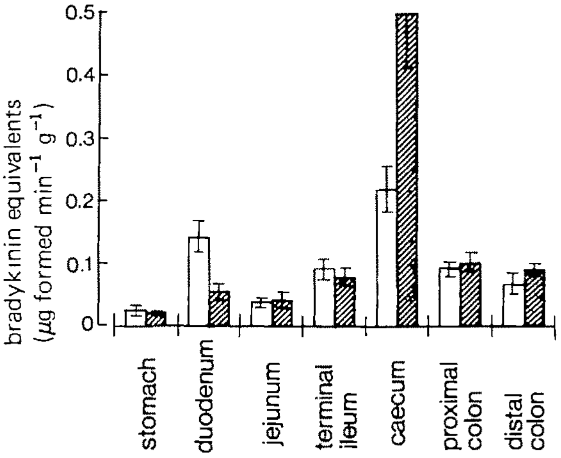

**Figure 1** Concentrations of kallikrein in rat intestine, expressed as µg bradykinin equivalent formed min$^{-1}$ gram$^{-1}$ wt of wet tissue. Open columns refer to starved animals while cross-hatching denotes normally fed rats. (Means of 6 rats ± s.e.)

This work was supported by a grant from the MRC to whom NHF is grateful for a studentship. We thank Sandoz Ltd. for a gift of synthetic bradykinin.

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# State-dependent effect of diazepam on learning

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State-dependent learning presents a methodological problem in studies concerning drug effects on memory processes. It can also be a prognostic indicator for psychiatric patients undergoing joint psycho- and chemotherapy. Moreover, several drugs capable of inducing strong state-dependent effects have the potential for abuse by man (Overton, 1972).

The present double-blind study with diazepam (10 mg) on 20 student volunteers attempted to assess state-dependent effects of diazepam, which otherwise impairs memory (Gregg, Ryan & Levin, 1974). The tests used were Kahn's Symbol Arrangement Test and a paired association learning task. The different phases in information processing were experimentally differentiated in order to observe the effects of diazepam on encoding, storage, and retrieval (Atkinson & Shiffrin, 1968).

Table 1 presents the main results analysed by a two-way analysis of variance. The interaction F value for means in the same state and change state indicates the state-dependent effect. A significant dissociation effect was found in paired association learning task ($P < 0.001$), but not in the Symbol Arrangement Test. The significance arose, when the PP condition changed to PD, and DD condition to DP condition. Interference was greater when the subjects were under the drug during the retrieval phase (groups PD and DD) as compared to the retrieval under placebo (groups PP and DP). Neither acute nor subacute treatment revealed a significant drug effect on the encoding phase, even if mean number of mistakes was greatly increased in acute diazepam condition. We conclude, that the change-state effect of diazepam was due to the action of drug on the recall phase, and that diazepam has two kinds of action: a slight acquisition impairing, and recall facilitating effect.

**Table 1** Drug effects on the state-dependent learning

| | *Number of correct responses; Mean ± s.d.* | | | | |
|---|---|---|---|---|---|
| *Task* | *PP* | *PD* | *DP* | *DD* | *F value* |
| *Symbol arrangements*<br>(number of objects correctly ordered) | 5.2 ± 1.8 | 5.2 ± 1.3 | 5.0 ± 1.3 | 3.5 ± 2.3 | 1.83 |
| *Paired associations*<br>(number of correct answers) | 3.3 ± 2.1 | 4.2 ± 1.3 | 3.6 ± 1.3 | 4.8 ± 1.0 | 7.99*** |

The analysis of positive transfer on four groups, 10 subjects in each. PP=placebo-placebo, PD=placebo-diazepam, DP=diazepam-placebo, DD=diazepam-diazepam; the first letter refers to treatment on day 1, and the second letter to the treatment on day 2. Paired associations refer to the correct answers given to the test syllables on the next day after the right answers had been learned on the first day. The interaction F values for means in the same state and change state indicate the state-dependent effect.

*** $P < 0.001$.

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## Transducer coupling to pen recorder

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Electronic transducers coupled to expensive recording polygraphs with interchangeable signal conditioning modules are often used to measure physiological parameters. A low cost Transducer Coupler Unit has been developed which is simple to use and avoids the need for interchanging of modules. The circuit is illustrated in Figure 1. It enables the coupling of any type of transducer used in physiological research to a standard pen recorder which does not have built-in signal conditioning facilities. The Coupler can be made as a multi-channel unit. Each channel has a variable 0–20V DC stabilized power supply, adjustable with a potentiometer to suit the excitation voltage requirement of the transducer. A two-way input selector switch is provided for either *bridge* or *direct* coupling of transducers to the pen recorder. When used in *bridge* position, a multi-turn potentiometer is provided to adjust with the ten-turn duodial for zero balancing, to permit the coupling of any transducer with combined bridge resistance between 50–1500 ohms. There is also a three-position switch for filtering out unwanted signals and a reverse polarity switch to record signals in either direction of the pen recorder chart. The Coupler is already in use in the Department of Pharmacology, at the Royal College of Surgeons in conjunction with blood pressure transducers, isometric strain gauge type and rotary capacitive reactance smooth muscle transducers.

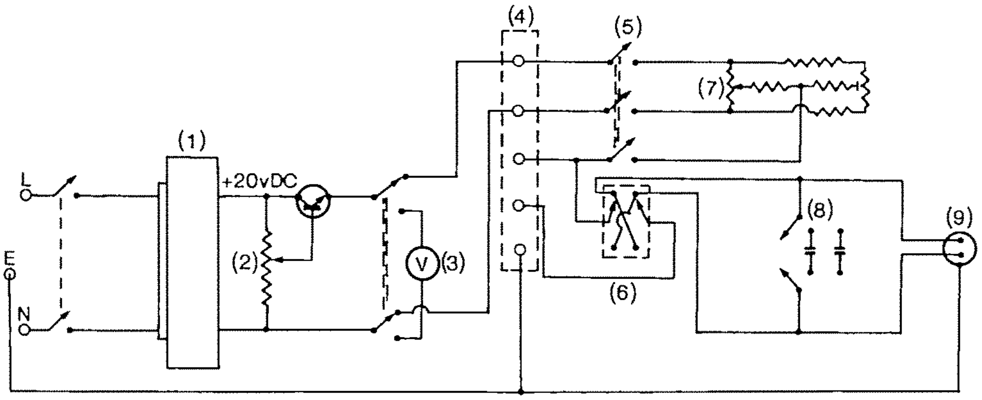

**Figure 1** 1. Power unit. 2. Adjustable excitation voltage control. 3. Voltmeter. 4. Transducer input socket. 5. Input selector switch. 6. Reverse polarity switch. 7. Balance bridge control. 8. Filter switch. 9. Output socket.

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## Measurement of the area under a curve (Planimeter)

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A low cost Biological Planimeter has been developed for use with mechanical digital counters. The DC output voltage of detectors used in laboratory experiments is converted into digital units proportional to the area under a curve of a pen recorder tracing. Examples of such tracings are those produced in gas chromatography and in various bioassays such as contractions of isolated tissues.

The circuit of the unit which is a modified integrator (Lockett, Carboni & Wilkins, 1976) is shown in Figure 1. It can be connected in parallel with a pen recorder and operates on 0–1 V DC positive, which is amplified to drive a totalizing counter with 60 pulses/second. There are only two controls. The first is the offset which is set to zero baseline of the recording, so that immediately the signal voltage at the input to the unit exceeds the offset point, the counting

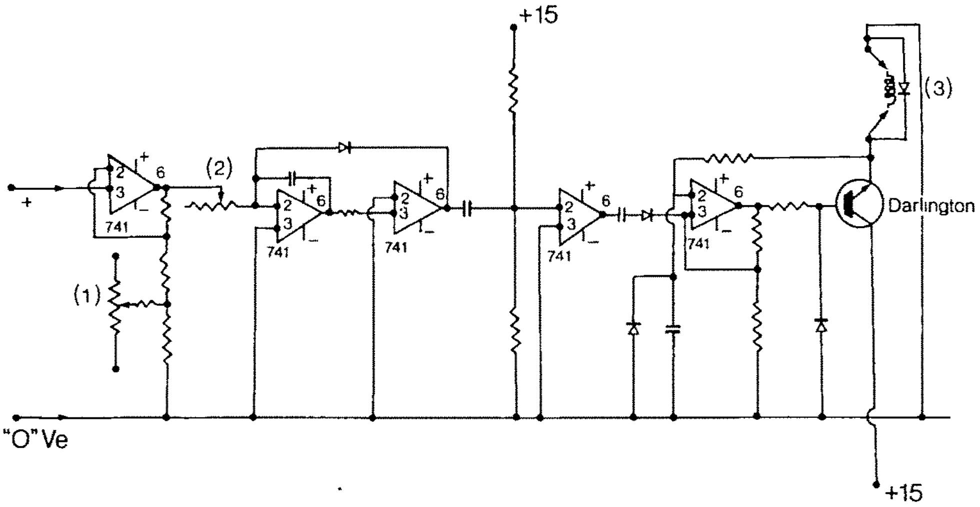

**Figure 1** 1. Set Zero Control. 2. Sensitivity control. 3. Coil of digital counter.

starts. The second is the sensitivity control which sets the counting rate. Both controls are adjusted with multi-turn potentiometers and ten-turn duodials. The Planimeter is at present used in the Department of Pharmacology, Royal College of Surgeons.

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## $^{31}$P and $^{1}$H n.m.r. studies of coenzyme binding to dihydrofolate reductase

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The coenzyme NADPH binds very tightly ($K_a > 10^8 M^{-1}$) to *L. casei* dihydrofolate reductase, and also substantially increases the affinity of the enzyme for inhibitors such as methotrexate. We have been studying the binding of coenzymes and coenzyme 'fragments' such as 2′AMP to the enzyme by $^{31}$P and $^{1}$H n.m.r. The 2′-phosphate group plays an important part in binding, and binds preferentially in the dianionic form. This preference is only 16-fold for 2′ AMP, but over 1000-fold for NADP$^+$ and NADPH. Together with changes in the chemical shift of the 2′-phosphate $^{31}$P resonance, this indicates that the environment of the 2′-phosphate in the complex is significantly altered by the binding of the rest of the coenzyme. The binding constant of 2′AMP, for example, may not therefore be an accurate measure of the contribution to binding of this part of the whole coenzyme.

Although NADPH binds three orders of magnitude more tightly than NADP$^+$, the $^{31}$P and $^{1}$H n.m.r. spectra show that the mode of binding of the adenine, 2′-phosphate and pyrophosphate moieties is closely similar for oxidized and reduced coenzymes. The large differences in binding energy can thus be localized to a difference in the interactions of the nicotinamide ring itself. However, binding of reduced nicotinamide mononucleotide is too weak to be detectable. The effects of coenzyme binding on the $^{1}$H n.m.r. spectrum of the protein show that binding of NADP$^+$ and NADPH (in contrast to that of 2′-AMP) is accompanied by conformational changes which differ for the two forms of the coenzyme.

The effects of inhibitors such as methotrexate or trimethoprim on the bound coenzyme are also largely localized to the nicotinamide ring binding site – changes in the $^{31}$P chemical shifts are minimal, but there is a large change in the $^{13}$C chemical shift of [carboxamido-$^{13}$C]-NADP$^+$.

## Co-operativity in ligand binding to dihydrofolate reductase

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The chemotherapeutic effectiveness of methotrexate arises from its extremely tight binding to dihydrofolate reductase. We are attempting to understand the interactions responsible for this tight binding in the case of *L. casei* dihydrofolate reductase. One potentially valuable approach to this problem is the study of the binding of 'fragments' of the ligands. The 'fragments' of methotrexate we have used are 2,4-diaminopyrimidine (DAP) and *p*-aminobenzoyl-L-glutamate (PABG), both of which bind relatively weakly ($K_a = 8 \times 10^3 \text{M}^{-1}$ and $1 \times 10^3 \text{M}^{-1}$, respectively). N.m.r. studies indicate that the fragments bind in a very similar way to the corresponding moieties of methotrexate. Not only do they bind simultaneously to the enzyme, but they bind *co-operatively*. Thus PABG binds 25-fold more tightly to the enzyme-DAP complex than to the enzyme alone. This co-operativity involves ligand-induced conformational changes in the protein, and indications of the amino-acid residues involved have been obtained from $^1$H n.m.r. The conformational changes resemble those accompanying methotrexate binding.

The binding and co-operativity of a series of analogues of PABG and DAP have been studied. The structural requirements for binding and for co-operativity are clearly distinct; for example, N-valeryl-PABG binds some 70-fold tighter than PABG, but shows no co-operativity with DAP. Some of the differences in conformation of the complexes involved in these effects can be observed by $^1$H n.m.r.

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## Nuclear magnetic resonance studies of ligand binding to dihydrofolate reductase

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Studies of the binding of inhibitors such as methotrexate and trimethoprim to dihydrofolate reductase will be used to illustrate some of the n.m.r. methods for the investigation of small molecule binding to proteins, and the kind of information which can be obtained.

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## Analysis of data by nonlinear regression analysis

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Many of the models used by pharmacologists for fitting experimental data are nonlinear e.g. in enzyme kinetics, and binding constant and pK determination. The most accurate, simple and unbiased way to fit experimental data to such models is by direct least squares estimation of the nonlinear parameters using a computer. By this method self-consistent values can be determined for all the unknown parameters of the problems together with statistical estimates of their accuracy. It is possible to produce a single computer programme which is simple to operate, yet can solve a very wide range of problems, both simple and complex.

The on-line use of such a programme to analyse kinetic and binding curve data will be demonstrated.

## Kinetic studies of coenzyme binding to *L. casei* dihydrofolate reductase

SUSAN M.J. DUNN & R.W. KING
(introduced by A.S.V. BURGEN)

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The kinetics of binding of substrates and substrate analogues to dihydrofolate reductase from a methotrexate-resistant strain of *L. casei* are currently being investigated by the stopped-flow technique. Complex formation can readily be monitored by measuring changes in protein or ligand fluorescence associated with binding. Reaction traces are collected by passing the amplitude photomultiplier signal into a 200 point signal averager for temporary storage prior to data transfer to the disc of an HP 3000 computer. Kinetic constants are determined by non-linear regression and both raw data and computed curves are plotted so that any deviation from, for example, a single exponential can be checked.

When NADPH is the substrate, under pseudo-first-order conditions the reaction curve, as monitored by dihydrofolate reductase fluorescence, shows a fast quench whose rate depends on NADPH concentration and a much slower first-order quench of rate approximately 0.03 $s^{-1}$. For the second order binding process a rate constant of $1.5 \times 10^7$ $M^{-1}$ $s^{-1}$ has been obtained. The amplitude of the fast phase as a percentage of the total signal change is not independent of ligand concentration and the results obtained suggest the existence of at least two interconvertible forms of dihydrofolate reductase, to one of which NADPH binds preferentially. The slow quench observed appears to be a reflection of the rate of interconversion of the enzymic forms.

S.M.J.D. is an MRC student.

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## Axotomy causes loss of synaptic contacts and loss of muscarinic receptors in the hypoglossal nucleus

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Tritiated propyl benzilylcholine mustard ([$^3$H]-PrBCM) is a potent, irreversible muscarinic antagonist whose binding satisfies the basic criteria for receptor-specific labelling (Burgen, Hiley & Young, 1974). We have used [$^3$H]-PrBCM to demonstrate autoradiographically the distribution of muscarinic receptors in the rat brain. In the brain stem the hypoglossal nuclei are heavily labelled.

Unilateral hypoglossal nerve axotomies (by ligation or by resection of a segment of nerve) were performed under anaesthesia in a series of adult (180–220 g) female Wistar rats. At various survival times after axotomy the animals for the autoradiographic study were sacrificed, coronal sections of unfixed brain stem (12 μm thick) cut in a cryostat, mounted on slides and briefly prefixed in cold 0.1% glutaraldehyde. Preincubation was carried out for 15 min in Krebs-Henseleit medium at 30°C with or without $10^{-6}$ M atropine followed by addition of cyclized [$^3$H]-PrBCM (40 Ci/mmol) to a final concentration of 5 nM for a further 15 minutes. The incubation was terminated by post-fixation in Carnoy's fluid followed by several washes in absolute alcohol. The slides were dipped in a 1 : 1 dilution of Ilford G5 nuclear emulsion, exposed at 6°C, developed in Ilford Phen-X and counter stained with cresyl fast violet. Specific binding is defined as the atropine-displaceable component of [$^3$H]-PrBCM uptake. The ratio of specific to non-specific binding was ca 3 : 1.

In a separate series of animals, the brains were prepared for light and electron microscopy after fixation by perfusion with a mixture of 1% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer and embedding in resin.

The hypoglossal nuclei were examined at progressively increasing times after axotomy; three short-term changes were detected.

(1) The specific binding of [$^3$H]-PrBCM was reduced by about half at 7 days.

(2) The neuronal nuclei showed indentations and perinuclear Nissl aggregations typical of chromatolysis. In the neuropil the dendritic profiles were much reduced in diameter; this correlates well with the previously described dendritic retraction and attenuation (Sumner & Watson, 1971).

(3) There was almost a 50% reduction in the number of synaptic contacts by 7 days. In place of the pre-synaptic terminals the former post-synaptic surfaces were covered with thin lamellae of glial cytoplasm; this agrees well with the findings of Sumner & Sutherland (1973).

At longer survivals, if the axon re-establishes functional contact with an appropriate target tissue, these changes are reversed. The hypoglossal motorneurones re-acquire a normal complement of afferent synapses (Sumner, 1975a, 1975b) and our observations show that the muscarinic receptor also reappears.

The parallel behaviour of the muscarinic receptor and the synaptic changes in the hypoglossal nucleus raises the possibility that there may be some causal relationship between the disappearance of the receptor and the loss of synaptic contacts.

A.R. is grateful to the Medical Research Council for a research studentship.

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# Some morphine-like properties of a potent antinociceptive synthetic pentapeptide in relation to physical dependence in rodents

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A pentapeptide analogue of leucine-enkephalin, BW 180C (Tyr.D-Ala.Gly.Phe.D-Leu), has been shown to possess a similar profile to that of morphine in antinociceptive tests in mice and in behavioural tests in mice and rats (Baxter, Goff, Miller & Saunders, 1977). It was therefore of interest to determine the extent to which the peptide also resembled morphine in tests designed to predict abuse liability. It has already been reported that the peptides, methionine-enkephalin and $\beta$-endorphin, are capable of inducing tolerance and dependence in rats when given directly into the brain (quoted by Iversen & Dingledine, 1976).

Comparative data will be provided for the effects of the peptide and morphine in the following tests in mice and rats.

The drugs were administered intracerebroventricularly (i.c.v.) to mice by direct brain injection and to rats through a chronically implanted cannula in a lateral ventricle.

Anti-nociceptive studies undertaken in rats using standard tail flick (radiant heat) and tail pressure methods revealed that the peptide and morphine had a significant anti-nociceptive action at doses (i.c.v.) of $\geqslant$1.25 μg and $\geqslant$0.3 μg/rat respectively.

The ability of the drugs to substitute for morphine was investigated in rats which were made tolerant to and dependent on morphine using the method of Buckett (1964). Rats maintained on morphine (400 mg/kg i.p. twice daily) were withdrawn by withholding morphine for 40 hours. Both BW 180C and morphine (i.c.v.) were able to suppress the characteristic withdrawal signs (shaking and writhing).

In studies in mice using the naloxone precipitated jumping test it was found that the characteristic compulsive jumping was precipitated by naloxone (80 mg/kg s.c.) in mice injected 1 h previously with BW 180C (i.c.v.) and morphine (i.c.v. or s.c.). Higher doses of BW 180C were required to elicit naloxone-precipitated jumping following s.c. injection. In contrast a variety of non-opioid centrally acting substances failed to give a jumping response in this test.

We conclude that BW 180C resembles morphine in the two simple tests reported and thus synthetic opioid pentapeptides may present a potential for abuse liability in man. However, it remains to be determined whether such compounds may initiate and sustain self-administration.

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# Variable-rate intravenous infusions to mimic the blood levels of peptide hormones produced by physiological stimuli or subcutaneous injection

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*National Institute for Medical Research and National Institute for Biological Standards and Control*

The extent to which peptide hormones are destroyed or metabolically altered at a subcutaneous site is being studied by measuring post-injection blood levels and reproducing them by i.v. infusion. The techniques involved, which are applicable to human subjects, will be demonstrated on freely-mobile dogs with indwelling catheters and miniature electrically-controlled syringe pumps. Similar methods are being used to study the relative contributions of various target tissues to the patterns of action of parathyroid hormone and insulin, administered by s.c. or rapid i.v. injection or by more physiological means.

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# A low-cost electronic teaching aid

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(Introduced by J.B.E. BAKER)

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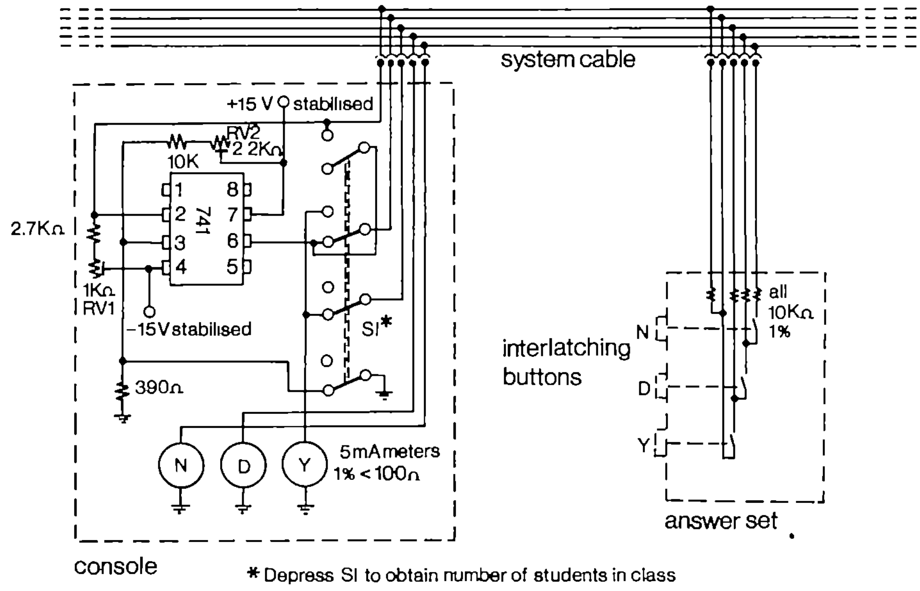
Multiple-choice-question (MCQ) techniques are a familiar feature of student courses; the electronic system demonstrated was designed for pharmacology MCQ's to provide the lecturer with an immediate indication of class answer percentages, and was installed by laboratory technical staff.

The system comprises (i) a number of simple hand-held answer sets, one of which is issued to each student for the duration of the MCQ test, each having three buttons marked Y (yes), N (no) and D (don't know); (ii) electrical sockets installed round the teaching room and interconnected by a single 5-way cable, into which the answer sets are plugged by the students; and (iii) a small electronic console, which is plugged into any of the above sockets, which calculates and displays on meters the percentage of



* Depress SI to obtain number of students in class

**Figure 1** Basic circuit diagram.

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[1] Present address: Department of Physics, Royal Marsden Hospital, Downs Road, Belmont, Sutton, Surrey.

students answering Y, N and D, and the total number of students (i.e. answer sets in use).

This is a high-resistance, low-current system (requiring a stabilized ±15 V supply) which works with 10–500 answer sets, is accurate to ±2.5% (±1% if hand calibrated), and costs only £3–£3.50 per student to install plus £35 for the console (including ±15 V supply). Following construction RV1 (Figure 1) is adjusted to give f.s.d. for 100%, and RV2 to calibrate the number-of-students scale on meter Y. The console may be upgraded for an additional £10 with extra circuitry to (i) improve the accuracy to 1% without the need for hand calibration, and (ii) to enable the console operator to 'freeze' the meter readings and eliminate any late answer changes which would otherwise alter the readings while being noted.

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## The determination of kynurenine by gas-liquid chromatography; evidence for its presence in rat brain

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Kynurenine is a major metabolite of tryptophan on a pathway initiated by the inducible liver enzyme tryptophan pyrrolase. This pathway is a route for the synthesis of NAD and it may compete with other pathways for available tryptophan, which may be limited. In particular competition with the synthesis of 5-hydroxytryptamine (5-HT) in the brain may be of importance in the precipitation of psychiatric depression (Curzon, 1969; Joseph, Young & Curzon, 1976).

The pyrrolase pathway has been studied in animals via the *in vitro* determination of tryptophan pyrrolase, and in animals and man via the urinary excretion of tryptophan metabolites, including kynurenine. Previously the relatively low sensitivity of analytical methods for the latter has required the use of loading doses of tryptophan. Recently we have described (Joseph & Risby, 1975) a more sensitive colourimetric method for the estimation of kynurenine in plasma (which requires the suppression of interference from tryptophan). The present demonstration shows that the *o*-amino acetophenone produced by heating kynurenine with strong alkali in that method can be conveniently and sensitively determined by gas liquid chromatography. Following extraction of the *o*-amino acetophenone into butyl acetate containing dichloro-*p*-xylene as internal standard it is derivatized with trifluoracetic anhydride; excess reagent is hydrolysed with 0.2M borax in 1.5M sodium hydroxide. 1 μl of supernatant is injected into a Hewlett-Packard 5713A gas chromatograph. Injection port 250°C, column 10% 0V1 on H.P. Chromosorb W at 120°C, detector 300°C $^{63}$Ni electron capture, carrier gas argon/methane (95/5) at 60 ml/minute.

When this method was applied to brain tissue from male Sprague Dawley rats (200–250 g) levels of some 200 ng/g kynurenine were apparent. The identity of the *o*-amino acetophenone has been confirmed by mass spectrometry and further evidence that it is derived from kynurenine will be discussed. Until recently it has been thought that tryptophan pyrrolase was not present in brain, but Gal (1974) has reported the existence of an enzyme in rat brain homogenate capable of synthesizing labelled kynurenine from labelled tryptophan. If the kynurenine present in brain is indeed synthesized there, there would be a possibility of competition between the 5-HT and pyrrolase pathways within the brain itself. These questions are the subject of current investigations.

I thank Dr A. Lawson of the Division of Clinical Chemistry, Clinical Research Centre for Mass-Spectrometric analysis of *o*-aminoacetophenone.

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# A superfusion chamber suitable for maintaining mammalian brain tissue slices for electrical recording

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Recent work has shown that mammalian brain tissue slices can be kept alive and electrically excitable *in vitro* (see Richards, Smaje & White, 1976). This technique is useful for the study of the mode of action of centrally-acting drugs because of the control one has over the environment of the nerve cells. However, earlier incubation chambers have generally placed the tissue slice at the interface between an oxygen-enriched atmosphere and the saline solution itself as this facilitates the recording of field potentials (Doré & Richards, 1974). The present apparatus was designed for the superfusion of both surfaces of the tissue slice thus permitting rapid changes in the bathing medium.

The apparatus consists of a small recessed chamber mounted on top of a thermostatically-controlled water bath which is kept at 38 ± 0.2°C (Figure 1). The tissue slice rests on a grid of platinum gauze just above the bottom of the recess and is prevented from floating by a nylon mesh. Solutions are prewarmed in tubes made of silicone rubber or nylon which are fixed to connectors on the top plate of the incubation assembly. To reach the tissue, the solutions pass through non-return valves made from 3.18 mm ceramic balls which seat on the top of the inner connectors. Only one solution is permitted to flow at any one time, its rate of flow being governed by the head of pressure applied. The solution is selected by a rotary switch which clamps five of the six tubes connecting the reservoirs of saline to the incubation chamber. The level of fluid above the slice is maintained by a suction pipette. A gas mixture of 95% $O_2:5\%\ CO_2$ is bubbled through the water bath before passing over the solution in the incubation chamber.

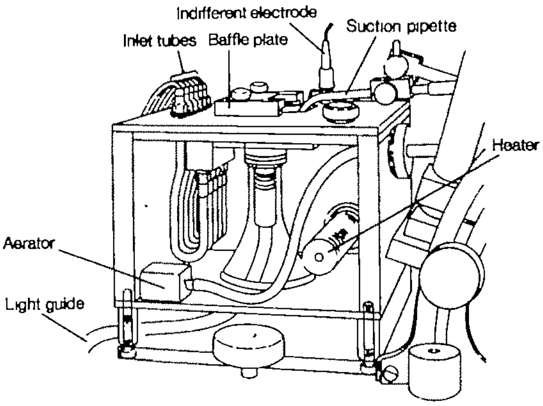

**Figure 1** A simplified three dimensional view of the apparatus.

This apparatus can also be adapted for other mammalian tissues and has the advantage of a very small dead space.

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# A novel and inexpensive method for detecting and quantitating the occurrence of cardiac arrhythmias

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The most time-consuming part of work aimed at finding anti-arrhythmic drugs is identification and quantification of normal and ectopic ECG complexes. Commercial equipment is expensive and, being clinically orientated, does not present data in a form ideally suited to experimental work.

This demonstration illustrates some variations of a novel method of recognizing normal (sinus) ECG complexes in the presence of multifocal ventricular dysrhythmias. The method entails the successive triggering (within a prescribed interval) of two (or more) phototransistors placed over the face of a display oscilloscope. A simple voltage *versus* time display of an ECG may be employed, but some ectopic QRS complexes can take the form of sinus complexes. Therefore, the preferred method uses triggering of the phototransistors by vectorcardiogram loops formed from ECG leads I and II, after suitable band-pass filtering to eliminate slow and fast ECG artefacts. Oscilloscope beam brightness modulation of suitable duration is also employed, and enables the display of complete normal vectorcardiograms, whilst limiting display of ectopics (wide QRS complexes) to shorter sections of loop. This helps reduce false recognition of ectopics as normals. A modification of this method may be employed when only one ECG lead is available. For equal amplitudes, normal ECG complexes have greater high frequency content, by virtue of their short duration, than do ectopic complexes, which are generally of long QRS duration. By filtering the single ECG signal through suitably adjusted high and low frequency band-pass filters, two signals may be derived. These signals will form X:Y display loops with sufficient difference between normal and ectopic loops to permit satisfactory normal complex recognition.

Using these methods of normal ECG identification, a computer has been developed which, whilst counting all ECG complexes, derives from the absence of normal complexes a count of ectopic beats. These data permit computation of mean ventricular rate, mean ectopic rate and percentage occurrence of ectopics, each over one minute intervals. Each result is inscribed in turn for 20 s of the succeeding minute on a single channel oscillograph.

These methods of analysis have been developed and used satisfactorily over the past three years to investigate the actions of known and novel chemical compounds.

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# Electron microscopic autoradiographic localization of [$^3$H]-dopamine in the dendrites of the dopaminergic neurones of the rat substantia nigra *in vivo*

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Previous studies from this laboratory suggested that the dendrites of the dopaminergic neurones in the substantia nigra of rat brain were capable of accumulating and releasing dopamine in a manner indistinguishable from that of dopaminergic nerve terminals in the corpus striatum (Geffen, Jessell, Cuello & Iversen, 1976). This evidence was, however, indirect and depended on the assumption that the sites of accumulation of exogenous [$^3$H]-dopamine in freshly microdissected slices of substantia nigra were predominantly in the cell bodies and dendrites of the dopaminergic neurones. Although this assumption is in agreement with the known morphology of the substantia nigra (Hajdu, Hassler & Bak, 1973) and is supported by fluorescence microscopy results (Björklund & Lindvall, 1975), it was possible that [$^3$H]-dopamine could be accumulated by an unsuspected population of dopaminergic or non-dopaminergic nerve terminals in the substantia nigra. This possibility has now been examined *in vivo* by the microinjection of 9.25 nmol of [$^3$H]-dopamine (specific activity 2.7 Ci/mmol) dissolved in 2 µl of artificial CSF, directly into the substantia nigra of rat brain from a stereotactically placed fine glass micropipette with a tip diameter of 25 µm. Fifteen minutes later the animal was killed by perfusion fixation with 5% buffered glutaraldehyde, and the substantia nigra prepared for autoradiographic examination as described previously (Kelly & Dick, 1976). Light and electron microscopic autoradiographs showed the autoradiographic activity to be predominantly localized over the dopaminergic cell bodies in the pars compacta and over their dendrites in the pars reticulata. Silver grains were only rarely seen over nerve terminals. Control experiments showed [$^3$H]-GABA to be accumulated solely by nerve terminals in the substantia nigra (Kelly & Dick, 1976).

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## A variable programme controller for sequential drug administration

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A solid state controller, using complimentary metal oxide semiconductor (CMOS) logic, has been developed for automating procedures in which drugs are administered at various times in one or two separate sequences, for example, with and without antagonist present. The unit can be programmed from potentiometers and switches on its front panel.

A schematic diagram is shown in Figure 1. The multiplexed clock controls the time between drug additions (1 min to 1 h) by altering the frequency of an oscillator which feeds into a binary counter. The counter output is fed into the appropriate sequencer which initiates the drug addition and selects the next drug's timing interval. At full capacity, 8 drugs can be administered sequentially in each of two independent channels (A and B). Up to 10 such channel cycles can be selected within a non-repeating programme and channels can be changed at the end of any preselected cycle. There is provision for introducing a delay of either 30 or 60 min when changing from channel A to B and this facility is useful, for instance, to enable antagonists to reach equilibrium with the tissue before any further agonists are added.

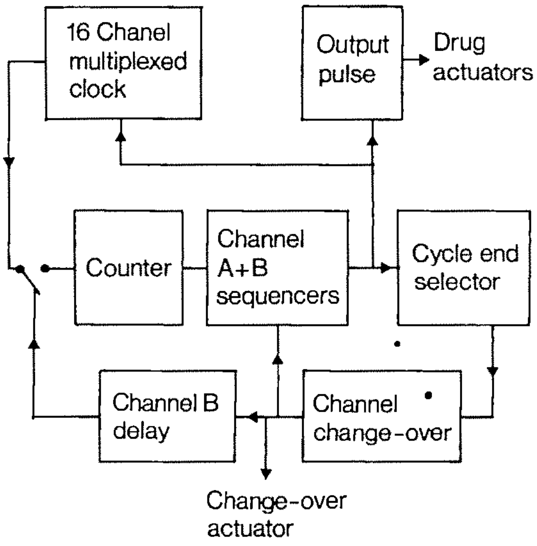

Figure 1

# A simple and inexpensive piece of apparatus for cascade superfusion procedures

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A variety of experimental systems have been designed for the cascade superfusion of tissues for investigations of biologically active substances (Armitage & Vane, 1964; Finkleman, 1930; Vane, 1964; Willis, 1969). The cascade arrangement is useful as it enables the simultaneous study of a variety of tissues.

Previous procedures have either used individual tissue baths and have carried out the experiments at room temperature or placed the apparatus in an air heated cabinet. The resulting apparatus is bulky, the heating arrangement complex and relatively expensive. The system which will be demonstrated was developed as a simpler and cheaper alternative.

The advantage of the method is that all tissues are kept in one water jacketed tissue bath (see Figure 1, A) which is heated by a conventional circulatory water pump to give an internal air temperature of 37°C. This therefore eliminates the need for a thermostatically controlled cabinet and use of individual tissue baths. The tissues are mounted on an easily constructed perspex tissue holder (B) into which a set of 5 tissue platforms (C) are inserted. Providing the platforms are kept in a fixed sequence they may be placed at any selected distance apart by inserting them into the angled grooves (D) cut into the vertical piece of perspex. The angle of the grooves, 20°, was found to be suitable for all purposes. The platforms are made from pieces of non toxic, non wettable plastic obtained from the outer containers of plastic syringes (Sherwood Medical Industries Ltd) and cut into a suitable shape and size. Each platform has a built-in hook (E) which serves as a tissue anchorage point and this hook is arranged vertically under the notch of the preceding platform. This ensures that the cotton thread to the levers/transducers from the tissue is not obstructed and also gives a suitable route of flow for the superfusing fluid.

Superfusion is therefore possible without the fluid coming into contact with any glass surfaces thus preventing drug absorption. Collection of the superfusate for analysis is possible by inserting a suitable plastic device in the last platform (F). The platforms are not restricted to tissues as plastic cylinders can be fabricated to take ion exchange resins for selective removal of active substances on their passage down the cascade.

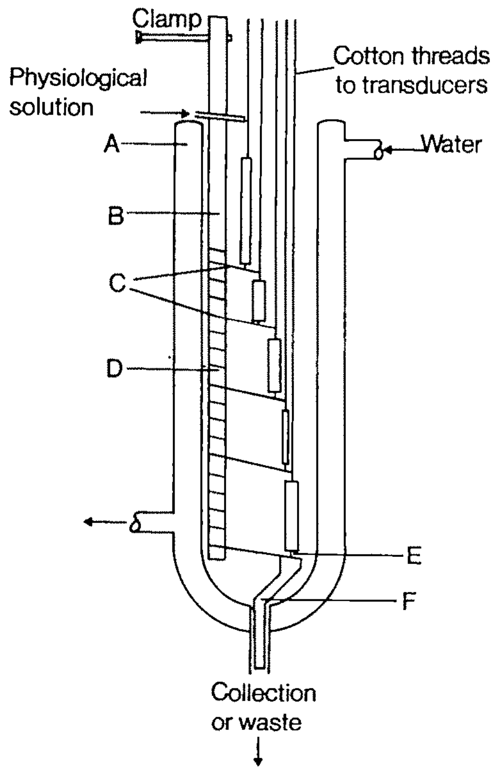

**Figure 1**

The method is now in routine use in our laboratory and it has been found that the tissues used in the cascade remain pharmacologically sensitive and stable for several hours.

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# An isolated guinea-pig working heart: preliminary studies with histamine and noradrenaline

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In 1965, Morgan, Neely, Wood, Liebecq, Liebermeister and Park, introduced an isolated, stable working heart from rats. This preparation has been used more extensively for biochemical and physiological studies (e.g. Neely, Liebermeister, Battersby & Morgan, 1967; Penpargkul & Scheuer, 1970) than for pharmacological studies.

The working heart preparation, unlike those based on the Langendorff preparation, performs external work. It also affords the measurement of aortic flow, left ventricular pressure and cardiac work in addition to sinus rate, force of contraction and coronary flow, thus allowing better characterization of changes in cardiac function. This preparation, unlike the heart-lung preparation, is completely isolated.

We have chosen to establish an isolated working guinea-pig heart as this species responds readily to cardioactive drugs such as histamine (Bartlet, 1963) and ouabain (Spilker, 1973).

Duncan Hartley guinea-pigs, of either sex, 450–600 g were killed 20 min after heparin (2000 u i.p.) and the heart excised. The aorta was cannulated and the heart perfused retrogradely for 2 min with Krebs Henseleit bicarbonate buffer equilibrated with 95% $O_2$/5% $CO_2$ at 37.5°C. During this time the left atrium was cannulated. When working, the heart was perfused via the left atrium at a pressure of 10 cm $H_2O$ and aortic flow was pumped against a pressure head of 70 cm $H_2O$. Drugs were injected into the left atrial cannula, just before the heart, at a constant volume of 0.5 ml over 5 seconds.

The parameters measured were sinus rate (S.R.), left ventricular pressure (L.V.P.), and its first derivative dp/dt, aortic flow (A.F.), coronary flow (C.F.), cardiac output (C.O.) and pressure work (P.W.). Where appropriate, measurements are expressed per gram dry weight of heart.

Preparations were left to stabilize for about 25 min and used for a further 45 minutes.

After stabilization, the values for parameters were: P.W. $0.326 \pm 0.013$ kg-m $min^{-1}$ $g^{-1}$, L.V.P. max. $85.7 \pm 1.5$ mmHg, dp/dt max. $1572 \pm 94$ mmHg/s, S.R. $211.3 \pm 4.1$ beats/min, A.F. $313.0 \pm 10.5$ ml $min^{-1}$ $g^{-1}$, C.F. $65.9 \pm 2.7$ ml $min^{-1}$ $g^{-1}$ and C.O. $371.7 \pm 11.8$ ml $min^{-1}$ $g^{-1}$ ($n = 9-16$).

Histamine injections between $1 \times 10^{-9}$ mol and $3.5 \times 10^{-8}$ mol produced dose-related increases in P.W., A.F. and C.O. and between $1 \times 10^{-9}$ mol and $1 \times 10^{-7}$ mol produced dose related increases in L.V.P. max. dp/dt max. and S.R. Effects on C.F. were small and variable. At histamine $3.5 \times 10^{-8}$ mol the increases were: P.W. $54.7 \pm 4.1$%, C.O. $23.3 \pm 7.2$%, S.R. $37.2 \pm 3.4$ beats/min, L.V.P. max. $51.7 \pm 13.4$%, dp/dt max. $127 \pm 22.2$%, C.F. $11.0 \pm 11.1$% and A.F. $24.5 \pm 6.9$% ($n = 3$).

Noradrenaline injections between $1 \times 10^{-10}$ mol and $1 \times 10^{-8}$ mol produced dose related increases in all measured parameters. At $1 \times 10^{-8}$ mol the increases were: P.W. $46.7 \pm 7.5$%, C.O. $29.9 \pm 7.4$%, S.R. $67.3 \pm 9.7$ beats/min, L.V.P. max. $28.0 \pm 2.7$%, dp/dt max. $138.6 \pm 21.0$%, C.F. $29.0 \pm 2.7$% and A.F. $30.8 \pm 8.9$% ($n = 3$).

These preliminary results indicate that the isolated working heart responds readily to cardiac stimulants and that this preparation is suitable for study of the effects of drugs on cardiac function.

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## A non-invasive method for the preparation of split brain rats using ultrasonics

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Severance of the corpus callosum after physical separation of the cerebral hemispheres can be performed in the cat (Myers, 1956) and the monkey (Downer, 1959). This method is not ideally suited to the rat due to the damage caused to the sinuses and the very soft cerebral hemispheres. Using a shielded-knife, good results have been obtained by Goodman & Russell (1974). However, such methods cause unavoidable neural damage in the process of inserting the cutting device through tissues on approaching the corpus callosum.

An appropriate alternating voltage supplied to a concave piezoelectric crystal produces ultrasonic waves which pass through a fixed focal point dissipating sufficient energy at that point to cause a lesion (Johnston & Dunn, 1976). Thus, ultrasonics offer a non-invasive approach and cleavage of the corpus callosum can be achieved by a series of small overlapping lesions.

Male albino rats (190–220 g) under chloral hydrate anaesthesia (500 mg/kg, intraperitoneally) were placed in a head-holder. A midline incision was made in the scalp, the underlying membranes scraped away from the skull, and a 4 mm hole trephined at a skull landmark (lambda). A rectangle of bone approximately 10 mm long and 4 mm either side of the midline suture was then removed to expose the cortex. The ultrasonic transducer was held in a micromanipulator and coordinates of the corpus callosum determined from the intersection of the sagittal and transverse sinuses. Ultrasonic energy from the transducer is coupled into the cortex by saline (37°C) flowing through a hollow cone from the transducer and over the brain. A total acoustic power output of 2 W at 3 MHz at approximately 200 W/cm$^2$ is used. Approximately spherical lesions of 0.9 mm diameter are produced with a 14 s exposure. Lesions can be observed in 100 µ sections prepared using a vibratome from brains removed 48 h later.

This method has the advantage of reaching deep-lying structures without cutting surrounding tissues and is of special importance where any extra damage may be harmful or make interpretation of results difficult.

This study was made possible with the generous collaboration of M. Halliwell (Ultrasonics Dept., Bristol General Hospital) to whom special thanks are given. We gratefully acknowledge the assistance of P. Wells, also of Bristol General Hospital and of H. Griffith and E. Brownell of Frenchay Hospital, Bristol.

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## Effects of long-term treatment with contraceptive steroids on plasma and brain tryptophan, brain 5-hydroxytryptamine, and locomotor activity in female mice

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Although contraceptive steroids may have marked effects on monoamine metabolism and behaviour, little work has been carried out on animals with normal ovarian function. We have studied effects of prolonged daily injections of norethistrone acetate (200 µg/kg) alone and in combination with ethinyl oestradiol (100 µg/kg) compared with daily vehicle injection. Locomotor activity (Animex activity meter) was determined continuously for 2 oestrus cycles prior to injection, then every 7th day throughout 42 days of treatment. Free and total plasma tryptophan and brain tryptophan and 5-HT were determined on the 43rd day (Bender, Boulton & Coulson, 1975; Denckla & Dewey, 1967; Curzon & Green, 1970) and compared with dioestrus values.

Biochemical results are shown in Table 1. Locomotor activity declined after both treatments. On the final day of treatment the activity of the

**Table 1** Plasma and brain tryptophan and brain 5-HT levels in dioestrus and hormone-treated female mice

| | *Dioestrus* | *Norethistrone* | *Norethistrone and oestradiol* |
|---|---|---|---|
| Plasma total tryptophan (μg/ml) | 24.2 ± 1.9 | 26.0 ± 0.5 | 22.0 ± 0.6* |
| Plasma free tryptophan (μg/ml) | 12.2 ± 1.3 | 15.2 ± 0.3 | 8.91 ± 0.44* |
| Brain tryptophan (μg/g) | 2.41 ± 0.20 | 2.60 ± 0.09 | 3.02 ± 0.47 |
| Brain 5-HT (ng/g) | 624 ± 21 | 632 ± 18 | 867 ± 12** |

Mean ± s.e. mean.
* Significantly different from norethistrone alone $P < 0.001$.
** Significantly different from both norethistrone alone and dioestrus values $P < 0.001$.

norethistrone group was 47% while that of the norethistrone plus ethinyl oestradiol group was 54% of initial dioestrus values. Vehicle controls resumed cyclic changes in locomotor activity within 8 days, while the hormone treatments abolished these cyclic changes.

The ineffectiveness of norethistrone in changing brain tryptophan is perhaps surprising in view of the acceleration of 5-HT turnover reported after progestogen treatment (Ladisch, 1974). The reduction in plasma total and free tryptophan on combined treatment with oestradiol may be due to acceleration of protein synthesis by the latter (Hamilton, Widnell & Tata, 1968). The rise in brain 5-HT could also be due to changes in storage following increased protein formation in nerve endings or perhaps to inhibition of monoamine oxidase by oestradiol (Morrison & Pritchard, 1971).

These findings do not support the hypothesis that brain tryptophan levels and hence 5-HT synthesis may be controlled by plasma free tryptophan (Knott & Curzon, 1972), since both brain tryptophan and 5-HT were raised by combined treatment, while plasma levels were lowered. The functional activity of these raised 5-HT levels is, however, in question since 5-HT has been suggested to be involved in mechanisms inhibitory to spontaneous locomotor activity (Neuburg & Thut, 1974; Grabowska & Michaluk, 1974), yet this activity declined to approximately the same extent in both treatment groups.

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## Recording foetal breathing in man

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Foetal lambs in utero make foetal breathing movements (FBM) which coincide with periods of rapid eye movement sleep (Dawes, 1974). This association and their suppression by phenobarbitone (Boddy, Dawes, Fischer, Pinter & Robinson, 1976), suggests that FBM are an indicator of arousal in the foetus. We are investigating FBM in man as a possible *in vivo* test system for measuring the central nervous depressant effects on the foetus of drugs, particularly the analgesics given to women in labour.

FBM have been recorded in man using A scan ultrasound (Boddy & Robinson, 1971). Movements of the foetal chest wall are detected by an ultrasonic beam. However, a variety of artefacts may be caused by other movements of the foetal chest relative to the ultrasonic beam (Farmer, Thomas & Blackwell, 1975). We have recorded FBM more directly, visualizing the foetus by real time ultrasound. The real time ultrasonic scanner (ADR, Arizona) has 64 separate transducers so arranged that each contributes a line of B scan to the image display, producing a moving picture of the foetus in cross section. The transducer array is moved across the maternal abdomen until the foetal chest and upper abdomen are visualized in sagittal section lateral to the spine and traversing the heart. FBM can be recognized as characteristic tipping movements as the anterior chest wall moves inwards while the anterior abdominal wall moves outwards. The image is recorded on video tape. No attempt has been made to register FBM electronically since it is only by direct observation that the other movements of the chest wall can be distinguished. These other movements include transmitted pulses from the maternal aorta, maternal respiration, foetal body movement (FM) and maternal movements. Records of FM and FBM are made by an observer using a manual tachometer.

Six women with uncomplicated first pregnancies were studied on 2 occasions between the 36th and 38th week of gestation. Recordings were made for 30 min between 8 and 9 a.m. following a 25 g glucose drink. The percentage of time during which the foetus made FBM and FM is shown in Table 1. No FBM could be distinguished during whole body movements. There was a tendency for individual foetuses to behave similarly on the two occasions. The average mean frequency of FBM was 52 ± 4/min (mean ± s.e. mean) with a range of 32 to 72/minute.

**Table 1** Occurrence of foetal breathing and foetal movements in six human foetuses studied on two occasions

| | *FBM: % time occurring* | | *FM: % time occurring* | |
|---|---|---|---|---|
| *Patient* | *1st time* | *2nd time* | *1st time* | *2nd time* |
| 1 | 90 | 93 | 3 | 3 |
| 2 | 62 | 72 | 21 | 16 |
| 3 | 51 | 48 | 6 | 2 |
| 4 | 13 | 66 | 11 | 17 |
| 5 | 82 | 57 | 20 | 6 |
| 6 | 25 | 15 | 18 | 24 |
| Mean ±s.e. mean | 54 ± 13 | 59 ± 11 | 13 ± 3 | 11 ± 4 |

Supported by the Wellcome Trust and the Cilag-Chemie Foundation.

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# Inhibitory actions of adenine nucleotides and adenosine on transmission in rat vas deferens

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During nerve stimulation, adenosine 5′-triphosphate (ATP) is released with catecholamines from the adrenal medulla (Smith & Winkler, 1972) and probably also with noradrenaline (NA) from adrenergic nerve endings (Burnstock, 1976). The physiological role of ATP or its metabolites following release is unclear (Burnstock, 1976). ATP inhibits release of acetylcholine from cholinergic nerves (Sawynok & Jhamandas, 1976) and adenosine may exert a similar presynaptic inhibitory action on noradrenergic neurones (Hedqvist & Fredholm, 1976).

We have examined the effects of ATP, adenosine 5′-pyrophosphate (ADP), adenosine 5′-phosphate (AMP) and adenosine on isometric responses of rat vas deferens *in vitro*.

ATP ($10^{-6}$–$10^{-4}$M) and adenosine ($3 \times 10^{-6}$–$10^{-4}$M) inhibited responses to field stimulation (150 pulses, 5 Hz, 1 ms, supramaximal voltage) in a dose-dependent manner, the initial 'twitch' response (Swedin, 1971) being more sensitive than the second, 'sustained' response (Figure 1). ADP ($10^{-4}$M) and AMP ($10^{-4}$M) also inhibited the responses and were equipotent with ATP but significantly less potent ($P < 0.05$) than adenosine. The inhibition was presynaptic in origin as motor responses elicited by exogenous NA ($5 \times 10^{-6}$M) were not altered by ATP or adenosine. The inhibition was not mediated indirectly by prostaglandin liberation as indomethacin ($3 \times 10^{-5}$M) had no effect on the inhibitory effects of ATP and adenosine.

Theophylline ($10^{-4}$M) inhibits the postsynaptic actions of adenosine (Burnstock, 1975) and also antagonized its presynaptic action in this preparation (Figure 1). The inhibitors of adenosine uptake (Stafford, 1966; Paterson, Kim, Bernard & Cass, 1975), dipyridamole ($10^{-5}$ M) and 2-amino-6-[(2-hydroxy-5-nitro) benzythio]-9-β-D-ribofuranosylpurine (compound 555) ($10^{-5}$M) significantly potentiated the effects of adenosine (Figure 1), and those of ATP, ADP and AMP.

These results indicate that ATP and its metabolites can inhibit the release of NA from noradrenergic nerves.

Compound 555 was kindly supplied by Dr A.R.P. Paterson, University of Alberta Cancer Research Unit. Supported by an Alberta Heart Foundation grant and a Canadian Heart Foundation Research Fellowship (A.S.C.).

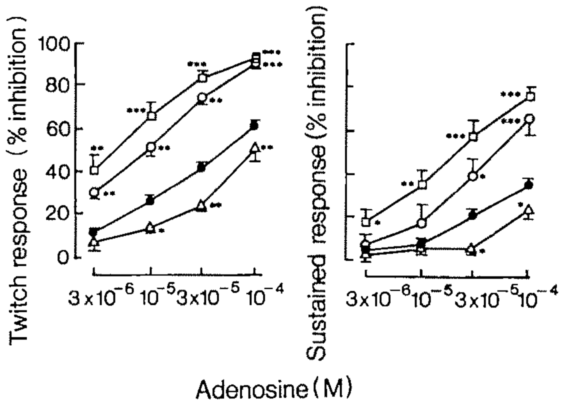

**Figure 1** Percentage inhibition (ordinates) of the 'twitch' (left panel) and 'sustained' (right panel) responses of rat vas deferens to field stimulation by different concentrations of adenosine (abscissae) in control Krebs (●, $n = 12$) and in the presence of theophylline ($10^{-4}$ M, △, $n = 6$), dipyridamole ($10^{-5}$ M, ○, $n = 6$) and compound 555 ($10^{-5}$ M, □, $n = 6$). Values are given as mean (±s.e. mean) for $n$ observations. The significance of the difference between control and experimental groups was estimated by the paired Student's $t$-test. Asterisks indicate: $^{*}0.05 > P > 0.01$; $^{**}0.01 > P > 0.001$; $^{***}P < 0.001$.

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# A unit for detecting, memorizing and recording peak voltages

J.A. MILSON
(introduced by J.F. MITCHELL)

*Department of Pharmacology, University of Bristol, Bristol BS8 1TD*

In experiments where multiphasic field potentials are continuously averaged and plotted on an analogue pen recorder, it is difficult to appreciate changes in amplitude of the component waves in the complex without manually plotting a graph of those amplitudes during each experiment. A unit was therefore devised which recorded the amplitude of each peak relative to the baseline and plotted out the results on a chart recorder. It was designed to be interfaced with the analogue output of an averager.

The analysing circuit (shown in part in Figure 1) makes extensive use of high input impedance operational amplifiers and digitally controlled solid state switches. All voltage holding circuits were chosen to be analogue rather than digital for simplicity, speed and accuracy. The circuit consists of a sample and hold amplifier recording the value of the pre-stimulus baseline which will be subtracted from the maximum field potential appearing at the output of the peak detector. The input signal is gated by the solid state switches to each of these sample and hold and peak detector circuit modules. The digital control circuit is designed in such a way that by easy external adjustment, these switches can be independently closed for carefully chosen periods during the analyser sweep time. The unit is currently used as a two-channel positive peak detector, but any combination of these circuit modules can be used so that negative or positive peak voltages can be recorded and referred to any other point in, or before, the waveform under investigation.

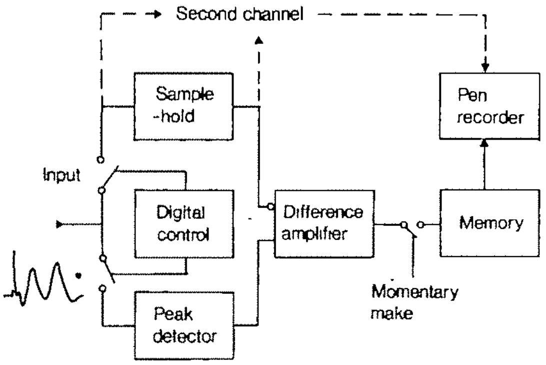

**Figure 1**

The level at the output of the difference amplifier is switched into the memory circuit at the end of a cycle and this level is held until the next sweep of the analyser. Drift rates of 6 mV/h (for signal inputs of ±3 V) can be achieved with care in printed circuit layout (Underwood, 1972). The output of the memory circuit is then presented to a pen recorder so drawing a graph of peak voltage values against time.

Although originally designed to interface with the output of an averager with an output sweep of 20 s, the circuit has proved to have a bandwidth of DC-300 Hz. It could therefore have considerable further uses in measuring, holding and recording peak voltage values of selected parts of any waveform within the limitations of its bandwidth.

## Reference

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# How to apply drugs to the ventral surface of the brain stem in anaesthetized cats (T)

W. FELDBERG & P.G. GUERTZENSTEIN

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# Does activation of 5-HT neurones support self-stimulation? (T)

T.J. CROW & J.F.W. DEAKIN

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Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

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*Br. J. Pharmac.* (1977), **59**, 539–549

# CHARACTERIZATION OF (±)-METHADONE UPTAKE BY RAT LUNG

C.H. CHI & B.N. DIXIT

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1 By use of a sensitive and specific fluorescence assay procedure it was shown that after subcutaneous administration to rats, (±)-methadone was concentrated in the lung. Lung to serum ratios ranging from 25 to 60 were obtained indicating that the rat lung tissue was capable of extracting (±)-methadone against a concentration gradient.

2 This phenomenon was investigated *in vitro* with rat lung slices incubated in Krebs-Ringer phosphate buffer (pH 7.4). The uptake was expressed in terms of tissue to medium concentration ratios (T/M ratio).

3 The principal observations were: (i) Studies on the time-course of the uptake showed that the T/M ratios of (±)-methadone increased rapidly during the first 60 min of incubation and then more slowly, with a plateau occurring at 180 min; (ii) The T/M ratio of (±)-methadone progressively increased from 9.5 to 17 as the pH of the incubation medium was varied from 6.2 to 7.8; (iii) When the concentration of (±)-methadone in the incubation medium was varied from 0.005 to 0.5 mM, the T/M ratio decreased rapidly suggesting self-saturation of the transport process. Beyond the medium concentration of 0.5 mM, the T/M ratio declined very slowly.

4 These results suggested that at low concentrations, (±)-methadone was transported predominantly by a self-saturable process while at higher concentrations it was transported by a process of simple diffusion.

5 At low concentrations (0.01 mM) the uptake of (+)-methadone was higher than that of (–)-isomer indicating stereo-specificity of the uptake process. The uptake of (±)-methadone at low concentration (0.01 mM) was significantly inhibited by low temperature, lack of $O_2$, lack of glucose, lack of $Na^+$ in the incubation medium, and by exposure of the tissue to high temperature (≈100°C). The uptake was also inhibited by relatively high concentration of iodoacetate (1.0 mM) and of naloxone (1.0 mM).

6 Kinetic analysis of data showed that the diffusion constant for (±)-methadone was 5.0 ($h^{-1}$) and the $V_{max}$ of the active transport process was 6.5 µmol $g^{-1}$ $h^{-1}$.

## Introduction

During the past decade, (±)-methadone has been extensively used in the maintenance and rehabilitation programmes for chronic heroin users (Dole & Nyswander, 1968). In spite of the work that has been done on the metabolic disposition of methadone (Beckett, Mitchard & Shihab, 1971; Pohland, Boaz & Sullivan, 1971; Misra, Mulé, Bloch & Vadlamani, 1973) there are still gaps in our knowledge about its absorption, distribution and metabolism.

By use of a specific fluorescence assay method, it was recently shown that after subcutaneous administration of (±)-methadone, high concentrations of the drug were found in the lung tissue of the rat (Chi & Dixit, 1973). This paper describes studies that were conducted to elucidate and characterize the mechanisms involved in this preferential distribution of (±)-methadone in the rat lung.

## Methods

### *Animals*

All experiments were performed with lung tissue slices obtained from male albino rats of Wistar strain (Hilltop Lab Animals, Scottdale, Pa.), weighing between 150 and 200 grams. They were housed in group cages (approximately 20 rats per cage) in animal rooms maintained at 23 ± 1°C with a 12 h 'light-dark' cycle. Water and food were provided *ad libitum*. At least three days were allowed for acclimatisation.

### *Fluorescence assay for (±)-methadone*

It was shown by McGonigle (1971) that when (±)-methadone reacts with paraformaldehyde in concentrated $H_2SO_4$ by heating at 100°C, a fluorophore is formed. Based on this observation, a fluorescence assay was developed. It will be briefly described here.

Rats were killed by decapitation, the blood and other tissues were removed and were frozen and stored at −20°C until assayed. Just before assay, tissues were weighed and homogenized in distilled water (1:3). A 2 ml aliquot of the homogenate or 1 ml of serum (or plasma) diluted to 2 ml with distilled water was used for the assay. One ml of $ZnSO_4$ solution (5%, $ZnSO_4.7H_2O$) was added to each sample and the contents were thoroughly mixed before the addition of 1 ml $Ba(OH)_2$ solution (4.5%, $Ba(OH)_2.8H_2O$). Aliquots (2 ml) of the supernatant were transferred into 15 ml glass stoppered centrifuge tubes, and 0.4 ml borate buffer, pH 9.6, was added to give the final pH of 9.2. Six ml of 25% isobutanol in ethylene dichloride was added and the tubes were shaken for a minimum of 8 min to facilitate the extraction of methadone into the organic phase. Three ml or any other suitable aliquot of the organic phase was evaporated to dryness at 50–55°C by using a jet of water-washed air; 0.1 ml of 0.1% paraformaldehyde in concentrated $H_2SO_4$ was added to each sample and the contents were heated in a boiling water bath for 15 minutes. Distilled water (7.1 ml) was added to adjust the final normality of the solution to 0.5; 30 min later the fluorescence was read on an Aminco-Bowman Spectrophotofluorometer with a photomultiplier opening No. 5, an excitation wavelength of 275 nm and an emission wavelength of 450 nm (uncorrected). Tissue blanks, using tissues of untreated animals, and a set of standards were run simultaneously with each set of unknowns. With this method 50 ng of (±)-methadone/sample could be accurately determined.

The specificity of the extraction procedure was established by determining distribution ratio profiles (Brodie & Udenfriend, 1945) of authentic (±)-methadone and methadone extracted from the homogenates of tissues obtained from rats that were treated with (±)-methadone. It was found that the extraction procedure is specific for (±)-methadone, the two principal metabolites of (±)-methadone (metabolite I and metabolite II) do not form a fluorophore when reacted with the paraformaldehyde reagent.

### *Tissue levels of (±)-methadone*

(±)-Methadone, 20 mg/kg, was administered subcutaneously to four groups of rats. They were killed 30, 60, 120 and 240 min after the injections. Serum, brain, liver and lung tissue samples were obtained and were analyzed for (±)-methadone.

### *Tissue uptake determination*

(i) *Incubation medium and drug solutions:* Krebs-Ringer phosphate buffer was used as the incubation medium and was prepared as described by Umbreit, Burris & Stauffer (1971) with the modification that only 1/5 as much phosphate buffer was used to buffer the solution. The pH of this solution remained unchanged (7.4) for as long as 3 h of incubation at 37°C. The solution contained 2 mg/ml of glucose. Drugs were dissolved in Krebs-Ringer phosphate buffer or 0.9% w/v NaCl solution. The pH of all drug solutions was 7.4. Appropriate volumes of these solutions were added to the incubation medium.

(ii) *Incubation procedure:* Rats were killed by decapitation. The lungs were immediately removed and were placed in oxygenated ice-cold Krebs-Ringer phosphate buffer. Small slices (18–20 mg) were cut with a pair of iris scissors. They were pre-incubated for 15 min in Krebs-Ringer phosphate buffer at 37°C. At the end of the pre-incubation period they were transferred to another set of flasks, each containing 15 ml of Krebs-Ringer phosphate buffer. The incubating medium contained appropriate concentrations of (±)-methadone.

All incubations were carried out in a Dubnoff Metabolic Shaker under $O_2$ (gas flow, approximately 1000 ml/minute). Unless otherwise specified, the incubations were carried out at 37 ± 1°C. Depending on the experiment, the period of incubation varied from 2 to 180 minutes. Whenever effects of other drugs, chemicals or metabolic inhibitors were studied, the lung slices were pre-incubated with the respective drug or chemical for 15 min and (±)-methadone was added to start the reaction. In some experiments $O_2$ was replaced with $N_2$. (±)-Methadone was added after the tissue was exposed to $N_2$ for 15 min, and the incubation was continued under $N_2$.

After incubation, the tissue was removed from the medium, rinsed at least twice in fresh ice-cold Krebs-Ringer phosphate buffer and was gently blotted on a piece of Whatman filter paper (No. 3) to remove excess medium. The tissue was homogenized in 5.0 ml glass distilled water using a Polytron ultrasonic homogenizer (Type P.T. 20, OD Kinematica, GMBH). A suitable aliquot of the aqueous homogenate was used for the determination of (±)-methadone.

### *Extracellular space determination*

Lung slices were incubated for various intervals and the incubating medium contained 0.01 mM of (±)-methadone and 0.5 μCi/ml of [$^{14}$C]-inulin. After incubation, each piece of tissue was removed from the medium, streaked on a piece of glass to remove excess medium (Lorenzo & Spector, 1973) and was weighed. The tissue samples were then dissolved in 1 ml of Soluene-100 in glass scintillation vials; 15 ml of toluene counting fluid was added to each vial and samples were counted for 10 min in a Packard Tri-Carb liquid scintillation spectrometer (Model 3310). To determine the [$^{14}$C]-inulin concentration in the medium, a 0.1 ml aliquot of the corresponding incubation medium was placed in similar vials and 15 ml of

Triton counting fluid was added. [$^{14}$C]-Inulin concentration was determined as above.

*Total tissue water determination*

Pieces of lung tissue were spread on watch glasses and weighed. They were dried to a constant weight at 100°C in an oven. The difference between the wet-weight and the dry-weight gave the total tissue water.

*Expression of results and statistical analysis*

The tissue concentration of (±)-methadone was expressed as µmol/g (wet weight). Tissue to medium concentration ratios (T/M ratios) were obtained by dividing the tissue concentration (µmol/g) by the medium concentration of (±)-methadone (µmol/ml). The uptake was expressed in terms of T/M ratios. The estimation of methadone in different tissues was done in duplicate. Group means, standard deviations and standard errors as well as Student's *t* scores were computed on an Olivetti-Programma 101 desk computer.

*Chemicals*

Sources of chemicals were as follows: all chemical reagents (A. R. or spectranalyzed grade) were from Fisher Scientific Co., Pittsburgh, PA 15238; (±)-methadone hydrochloride was from Mallinckrodt Chemical Co., Jersey City, New Jersey, 07303; the (+)- and (−)-isomers were kindly supplied by Eli Lilly Laboratories, Indianapolis, Indiana.

## Results

In vivo *studies*

Figure 1 shows the time-concentration curves for the lung, liver, brain and serum after 20 mg/kg (s.c.) of (±)-methadone was administered to rats. It can be seen that the level of (±)-methadone in lung tissue is several-fold higher than that of the serum. For example, at 30 min the level in the lung was 54.2 ± 6.7 µg/g (mean ± s.e. mean) and the serum level was only 1.33 ± 0.12 µg/ml (mean ± s.e. mean) resulting in a lung/serum ratio of 41. The lung/serum concentration ratios (mean ± s.e. mean, $n = 4$) at 30, 60, 120 and 240 min after 20 mg/kg of (±)-methadone were 40.5 ± 2.8, 43.0 ± 3.7, 54.8 ± 4.5 and 26.0 ± 3.7, respectively.

In vivo *studies*

The high lung/serum ratios of (±)-methadone obtained during *in vivo* experiments indicated that the rat lung tissue is capable of concentrating (±)-methadone against a concentration gradient. This phenomenon was investigated by conducting *in vitro* experiments with rat lung slices. The uptake of (±)-methadone by the rat lung slices was expressed as concentration ratio between the tissue concentration of (±)-methadone (T, µmol/g, wet weight) and the concentration of (±)-methadone in the incubation medium (M, µmol/ml). The final concentration of (±)-methadone in the medium was not determined in all experiments since the concentration of (±)-methadone was considered unaltered at the end of the incubation period as the volume of the incubation medium (5 ml) was much greater than that of the tissue employed (18–22 µl). Thus, in calculating the T/M ratio, the initial concentration of (±)-methadone in the incubation medium (M) was used.

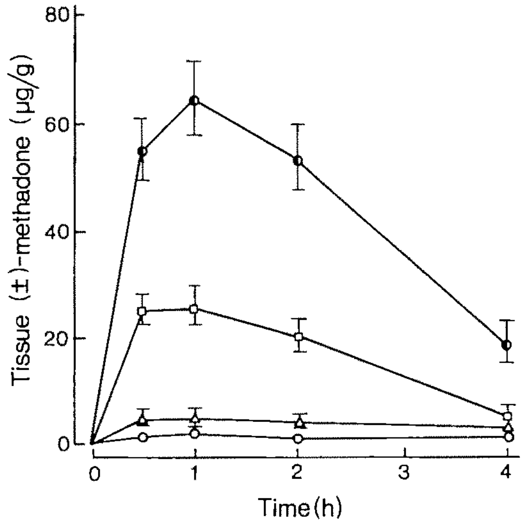

**Figure 1** Concentrations of (±)-methadone in serum (O), liver (□), brain (△) and lung (●) in rat. (±)-Methadone, 20 mg/kg, injected subcutaneously at zero time. The individual points represent mean values obtained from at least 4 animals. Vertical bars indicate s.e. mean.

*Time-course of uptake*

A 15 min pre-incubation of the lung slices had no significant effect on the uptake of (±)-methadone. The 60 min T/M ratio for pre-incubated tissue was 19.55 ± 2.04 (mean ± s.e. mean, $n = 3$); for control tissue it was 18.02 ± 1.52 (mean ± s.e. mean, $n = 4$).

It can be seen from Figure 2 that the T/M ratio increased rapidly up to 60 min of incubation and appears to reach a plateau in 180 min at which time the T/M ratio for (±)-methadone attained a mean value of 24 ± 2.67. [$^{14}$C]-Inulin T/M ratios were determined at 5, 15, 30 and 60 min of incubation. The [$^{14}$C]-inulin space was 30 ± 3.3% ($n = 3$) for a 30 min

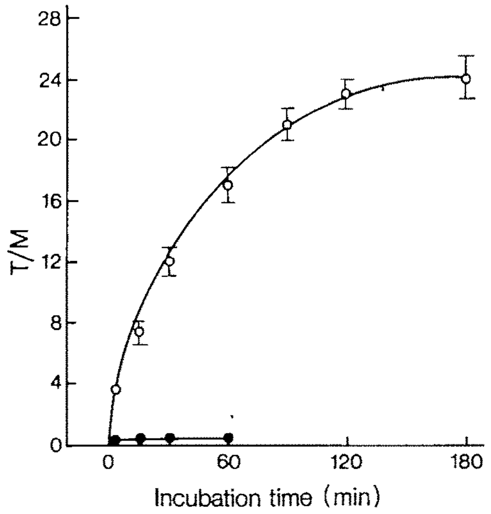

**Figure 2** Time course of the *in vitro* uptake of (±)-methadone (O) and [$^{14}$C]-inulin (●) by rat lung slices. T/M = lung tissue/medium (±)-methadone concentration ratio. Incubations under $O_2$ (gas flow, 1000 ml/min) at 37°C in Krebs-Ringer phosphate buffer, pH 7.4. (±)-Methadone concentration in the medium, 0.01 mM. [$^{14}$C]-inulin concentration in the medium, 0.1 µCi/ml. The individual points represent mean values obtained from at least 3 duplicate determinations. Vertical bars indicate s.e. mean.

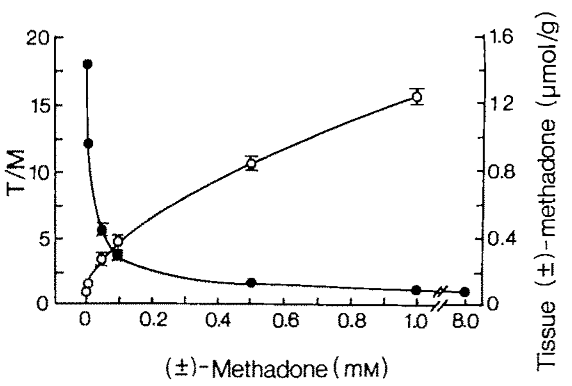

**Figure 3** *In vitro* uptake of (±)-methadone at various concentrations. The net uptake (O) and T/M ratios (●) are shown. T/M = lung tissue/medium (±)-methadone concentration ratio. Incubation under $O_2$ (gas flow, 1000 ml/min) at 37°C for 30 min in Krebs-Ringer phosphate buffer, pH 7.4. The individual points represent mean values obtained from at least 4 duplicate determinations. Vertical bars indicate s.e. mean.

incubation period. Total tissue water content was 81.1 ± 0.1% ($n$ = 10).

*Effect of pH of the incubation medium*

When incubations were carried out at pH 6.2, 6.6, 7.0, 7.4 and 7.8 for 30 min, and at M = 0.01 mM, the resultant T/M ratios (mean ± s.e. mean, $n$ = 3) were 9.3 ± 1.7, 9.5 ± 0.7, 11.3 ± 0.5, 13.2 ± 1.3 and 16.1 ± 1.5, respectively. In the present study all experiments were performed at pH 7.4 which is within the normal physiological limits of the pH of the extracellular fluid.

*Effect of concentration on the uptake*

As the concentration of the (±)-methadone in the incubation medium was increased from 0.005 to 32 mM, the T/M ratio at first decreased very rapidly and then approached near unity at higher concentrations (Figure 3). The T/M ratios for the medium concentration beyond 8.0 mM are not shown in the figure.

At the (±)-methadone concentration of 32 mM in the incubation medium, the observed T/M ratio was not significantly different from the one obtained when osmolarity was maintained by decreasing $Na^+$ and $Cl^-$ in the incubation medium (0.74 ± 0.1 and 0.72 ± 0.01, respectively). This indicates that the presence of 32 mM (±)-methadone in the medium does not affect the uptake characteristics of lung tissue due to possible changes in osmolarity.

*The uptake of (+)- and (−)-isomers of methadone*

With M = 0.01 mM, the T/M ratios (mean ± s.e. mean, $n$ = 5) at 1, 3, 10, 30 and 60 min for (+)-methadone were 2.5 ± 0.5, 6.2 ± 0.6, 10.0 ± 1.5, 14.8 ± 0.9 and 19.9 ± 1.6, respectively. However, under similar conditions the T/M ratios for (−)-methadone were 1.7 ± 0.8, 3.4 ± 1.0, 6.3 ± 1.1, 8.1 ± 1.1 and 13.6 ± 0.7 indicating that the uptake of (+)-isomer was significantly higher than its (−)-isomer.

*Effect of temperature*

When incubations were carried out for 30, 60, 90 and 180 min at 0–4°C with M = 0.01 mM, the T/M ratios (mean ± s.e. mean, $n$ = 3) were 6.3 ± 0.6, 7.8 ± 1.3, 7.6 ± 1.9 and 7.8 ± 1.0, while at 37 ± 0.5°C the corresponding T/M ratios were 21.1 ± 1.7, 19.6 ± 2.0, 21.5 ± 2.0 and 23.8 ± 2.7. These data indicated that the uptake of (±)-methadone was temperature-dependent.

*Effect of anoxia*

When incubation was carried out under $N_2$ instead of $O_2$, the uptake was inhibited by 73%

(T/M = 12.5 ± 0.2 under $O_2$ and 3.4 ± 0.3 under $N_2$). Results obtained with 30 min incubation indicated that the degree of inhibition decreased as the concentration of (±)-methadone in the incubation medium was progressively increased. At and beyond the concentration of 0.1 mM no significant inhibition of uptake was seen. Increase in the duration of incubation beyond 30 min did not have any significant influence on the inhibition of uptake.

*Effect of lack of glucose*

Omission of glucose from the incubation medium reduced the T/M ratio from 12.5 ± 0.2 to 3.1 ± 0.6 resulting in a 70% inhibition of the uptake. The degree of inhibition in uptake was not dependent on the period of incubation.

*Effect of exposure of tissue to higher temperature*

When the lung tissue was boiled for 3 min at 100°C in a water bath before incubation the uptake was inhibited by 85%.

*Effect of iodoacetate*

At a concentration of 0.01 mM of (±)-methadone in the incubation medium, a 15 min exposure to iodoacetate (0.01 mM) did not have significant effect on the uptake of (±)-methadone, but at 1.0 mM iodoacetate, (±)-methadone uptake was inhibited by 23%.

*Effect of lack of $Na^+$*

When $Na^+$ in the incubating medium was partially (50%) replaced by $Li^+$ as LiCl, the uptake was inhibited by 36%. A complete replacement of $Na^+$ by $Li^+$ resulted in 76% inhibition of the uptake indicating that the (±)-methadone uptake was $Na^+$-dependent and this dependency was related to the degree of $Na^+$ deficiency.

*Effect of morphine and naloxone*

Morphine in concentrations up to 1.0 mM had no significant effect on the (±)-methadone uptake. Similarly, 0.01 mM naloxone was without significant effect; however, at 1.0 mM naloxone, (±)-methadone uptake was inhibited by about 45%. In both cases, (±)-methadone concentration in the incubation medium was 0.01 mM.

## Discussion

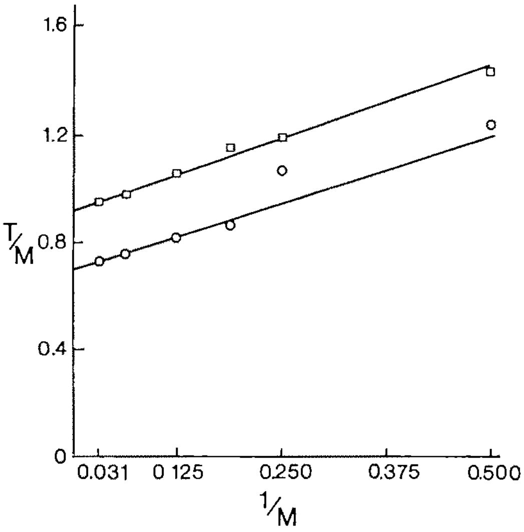

**Figure 4** Relationship between T/M and 1/M. T/M = lung tissue/medium (±)-methadone concentration ratio. Incubations for 15 min (○) and 30 min (□) at 37°C under $O_2$ (gas flow, 1000 ml/min) in Krebs-Ringer phosphate buffer, pH 7.4. (±)-Methadone concentration in the medium, 0.01 mM. Linear fit by T/M = b(1/M) + a; a = 0.71, b = 0.93 for 15 min incubation, and a = 0.91, b = 1.20 for 30 min incubation.

It was demonstrated *in vivo* that, after its subcutaneous administration, (±)-methadone was present in very high concentration in lung tissue of the rat. Lung to serum ratios ranged between 25 and 65, indicating that the rat lung is able to concentrate (±)-methadone against an apparent concentration gradient. The purpose of the *in vitro* experiments was to elucidate and characterize the mechanisms involved in this preferential *in vivo* distribution of (±)-methadone. *In vitro* incubation of lung slices seemed to be the most suitable procedure since it permitted detailed examination of the uptake characteristics and the factors that affect the uptake process. From the results obtained it can be safely concluded that (±)-methadone is taken up by the lung tissue by at least two processes: (i) At low concentrations in the incubation medium, (±)-methadone is taken up by a transport process that has the characteristics of an 'active transport' process. It was shown that (±)-methadone is taken up against an apparent concentration gradient; the transport process being inhibited by low temperature, lack of glucose, lack of $Na^+$ in the incubation medium and the presence of $N_2$ or the lack of $O_2$; (ii) At high concentrations of (±)-methadone, the major process of transport looks almost identical to that of 'simple diffusion' with the transport due to active process becoming nearly constant.

Studies on the time-course of the uptake showed that the T/M ratio of (±)-methadone increased more rapidly during the first 60 min of incubation and then more slowly, with a plateau occurring at 180 minutes. Since this is the first time that lung slices have been used to study the uptake of a drug, it is not possible to compare the results obtained in the present investigation with other studies. Most other studies have been conducted on isolated lungs of rabbits or rats that were perfused either with artificial media with or without added constituents of blood or with whole blood (Alabaster & Bakhle, 1970; Rosenbloom & Bass, 1970; Junod, 1972a, b; Iwasawa, Gillis & Aghajanian, 1973; Orton, Anderson, Pickett, Eling & Fouts, 1973). The development of oedema has been the most common difficulty encountered in perfusion experiments using artificial media. According to Nicolaysen (1971), Lunde (1967) and Orton *et al.* (1973) whole blood is the most suitable perfusing medium. In the present studies the weight of lung slices increased by about 13% at the end of 60 min of incubation indicating that oedema formation was not a significant factor. When the lung is perfused, the perfusate is forced through the capillaries because of the 'pressure gradient' which causes the fluid to accumulate in the extracellular interstitial space resulting in oedema formation. Such a 'pressure gradient' is not a factor when lung slices are incubated *in vitro* and might explain the lack of significant oedema formation observed in the present experiments. The observation, that neither a 15 min pre-incubation at 37°C, nor keeping the lung tissue at 0°C for as long as 3 h in the Krebs-Ringer phosphate buffer adversely affected the capacity of the lung tissue to concentrate (±)-methadone, can be interpreted as an indication that under these experimental conditions the lung slices remain viable for a considerable period of time.

It was shown that the T/M ratio of (±)-methadone increased from 9.5 to 17 as the pH of the incubation medium was varied from 6.2 to 7.8. Experiments with pH above 7.8 were not conducted since $Ca^{2+}$ and $Mg^{2+}$ begin to precipitate as phosphates. The observed effect of pH change on the (±)-methadone uptake can be interpreted in different ways. The pKa of (±)-methadone is 8.3–9.0(Misra, 1972). As the pH of the incubation medium was increased from 6.2 to 7.8, the proportion of unionized form of (±)-methadone increased from less than 0.2% to 33% (Table 1). Since unionized compounds generally diffuse easily across cell membranes (Davson & Danielli, 1952; Schanker, 1962) more (±)-methadone may have partitioned into the lung tissue. Inherent in this explanation is the assumption that the intracellular pH changes vary little during the period of incubation. Even if this were true, it cannot explain the magnitude of the uptake as indicated by T/M ratios of the order of 10–15. One could also suggest that (±)-methadone is transported only in the unionized form by an active transport process. This might explain the increase in the uptake as the pH of the incubation medium is increased. In view of the fact that the active transport process works against a concentration gradient, the latter explanation may not be entirely acceptable unless one shows that the concentration of the unionized (±)-methadone in the incabation medium is much lower than the apparent *Km* of the active transport process. A critical evaluation of the apparent *Km* of the active transport of (±)-methadone has not been done in the present investigation, however, the data shown in Figure 5 indicate that the *Km* would be of the order of 0.05 to 0.4 mM. This value is 5 to 40 times higher than the concentration of (±)-methadone used (0.01 mM) when studying the effect of pH on the uptake. Therefore, the difference between the concentration of the unionized (±)-methadone and the apparent *Km* will even be greater. It can also be speculated that the change in pH of the incubation medium affects the tissue metabolism and consequent energy production in a manner that adversely affects the active transport process. No experimental data are available to support such a mechanism.

When investigating the uptake of morphine by choroid plexus, Takemori & Stenwick (1966) observed that as the pH of the incubation medium was varied from 6.5 to 7.5, the T/M ratio of morphine increased from 2.5 to 9.0 and beyond the pH of 7.5 there was no further increase in the T/M ratio. It was observed by Hug (1967) that varying the pH of the medium over the range of 6.8–8.0 produced no consistant effect on the uptake (T/M ratio) of dihydromorphine by choroid plexus. These investigators were unable to draw any definite conclusions as to why the uptake of morphine was facilitated with increasing pH but that of dihydromorphine was unaffected. It must be mentioned that no systematic investigation has been done to evaluate the effect of extracellular pH change on the active transport processes.

When the concentration of (±)-methadone in the incubation medium was varied from 0.005 to 32 mM the T/M ratio decreased rapidly (Figure 3) suggesting self-saturation of the transport system which is one of the most important criteria of an active transport process. Beyond a certain medium concentration of

**Table 1** Effect of pH on the ionization of (±)-methadone

| *pH of incubation medium* | *(±)-Methadone (Ionized) / (±)-Methadone (unionized)* | |
|---|---|---|
| | *pKa=8.3* | *pKa=9.0* |
| 6.2 | 125 | 630 |
| 6.6 | 50 | 250 |
| 7.0 | 20 | 100 |
| 7.4 | 8 | 40 |
| 7.8 | 3 | 16 |

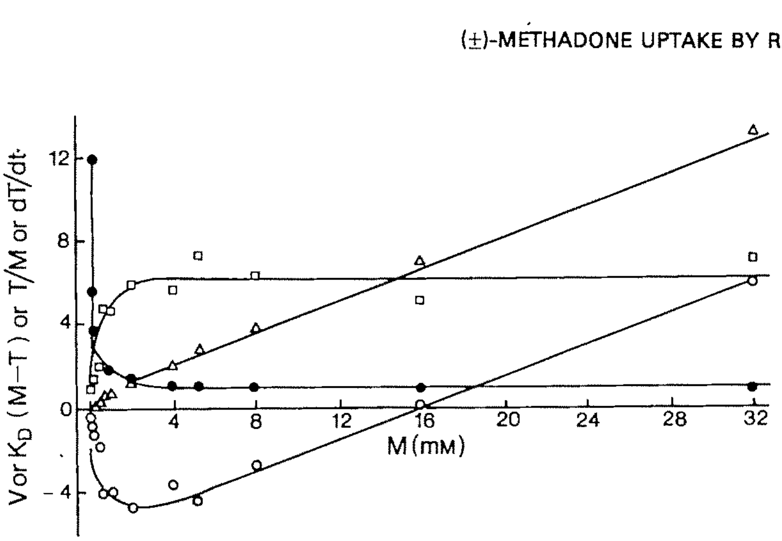

**Figure 5** Plots of V, μmol $g^{-1}$ $h^{-1}$ (□); $K_D$(M−T), μmol $g^{-1}$ $h^{-1}$ (○); T/M, lung tissue/medium (±)-methadone concentration ratio; or dT/dt, μmol $g^{-1}$ $h^{-1}$ (△) vs M (mM). Incubation period 30 minutes. All values except M are calculated. See text for details.

(±)-methadone (approximately 0.2 mM), the T/M ratio declined very slowly. If one looks at the relationship between the tissue concentration and the medium concentration, it is easy to realize that at higher concentrations (>0.5 mM), (±)-methadone is being transported by a process of simple diffusion. These results suggest that at low concentrations (±)-methadone is transported predominantly by a self-saturable process while at higher concentrations it is transported by a process of simple diffusion.

The observation that the uptake of (+)-methadone was higher than that of the (−)-isomer indicates that the uptake process shows significant stereospecificity thus fulfilling one additional criterion of an active transport process. Although Orton *et al.* (1973) investigated the uptake of (±)-methadone by isolated perfused lung of rabbit, data on stereospecificity were not reported. However, Hug (1967) demonstrated that the *in vitro* rate of uptake of dextrorphan by the rabbit choroid plexus was greater than that of levorphan. He suggested that the transport mechanism was the same for either compound, but the relative affinity of dextrorphan and levorphan were different. Stereospecificity has also been demonstrated for the adrenergic neuronal uptake (Uptake$_1$) of noradrenaline (Iversen, 1967) and for several amino acids by different tissues (Schultz & Curran, 1970). However, it was not possible to demonstrate stereospecificity for the uptake of noradrenaline (NA) by perfused rabbit lung (Iwasawa *et al.*, 1973). The present data are not complete enough to draw any conclusions about the differential kinetic characteristics of the uptake of the two stereoisomers of methadone.

Significant inhibition of (±)-methadone uptake at low temperatures (0–4°C) indicates the temperature-dependent nature of this uptake process. Other investigators (Junod, 1972b; Orton *et al.*, 1973) have also shown that lower temperatures adversely affect the uptake of NA and 5-hydroxytryptamine (5-HT) by perfused lungs of rabbits and rats with inhibition ranging from 54 to 85%. In the present study it was observed that the inhibitory effect of low temperatures is greater when the substrate concentrations are lower. This, of course, would be expected if one considers that these substances are transported by an active process followed by simple diffusion.

Elimination of $O_2$ from the gaseous phase significantly inhibited the uptake of (±)-methadone suggesting that the 'energy' for the transport mechanism was supplied largely by aerobic or oxidative metabolism. This was particularly true at low concentrations because it was observed that the degree of inhibition decreased as the concentration of (±)-methadone in the incubation medium was increased. This would be expected, if one takes into consideration that (±)-methadone is transported by two processes, the $O_2$-dependent active transport being mainly responsible at low concentrations and simple diffusion becoming increasingly important as the concentration of (±)-methadone is increased.

Omission of glucose from the incubation medium produced a significant inhibition of (±)-methadone uptake, the degree of inhibition being nearly constant throughout the entire incubation period. These data would suggest that the energy for the transport process is mainly derived from the oxidative metabolism of glucose. A small amount of endogenous glucose is likely to be present and is probably utilized by the tissue to provide some energy. The inhibitory effect is in sharp contrast to the findings of Junod (1972b) and of Iwasawa *et al.* (1973), who found that the lack of glucose did not have a significant effect on the uptake of 5-HT and NA by the isolated perfused lung of the rat. The results, therefore, would indicate that the site and the mechanism of (±)-methadone uptake may be different from those involved in the uptake of 5-HT and NA by the perfused lung. The results of Strum & Junod (1972) and that of Iwasawa *et al.* (1973) have shown that the uptake of NA and 5-HT occurs mainly in the capillary endothelium. At present, it is difficult to speculate on the site of the uptake of (±)-methadone in the lung tissue.

The need for adequate oxygenation and glucose availability indicated that the (±)-methadone uptake is an energy-dependent active transport process. This process is expected to perform optimally only under normal physiological conditions. When the lung slices were exposed for a very short period of time (3 min) to high temperature (≈100°C), the (±)-methadone uptake was drastically reduced. Such exposure to high temperature would disrupt all the 'energy' yielding processes and also the integrity of the cell membrane and the uptake observed is most likely to be due to simple diffusion. This observation provides indirect evidence to support the contention that (±)-methadone is taken up by the lung tissue by an active transport process.

The (±)-methadone uptake by the lung tissue was adversely affected when extracellular $Na^+$ concentration was reduced by replacement with $Li^+$ indicating that the transport process is dependent on extracellular $Na^+$. Junod (1972b) has recently shown that the uptake of 5-HT by the isolated perfused lung of rat is also adversely affected by a deficiency of $Na^+$ in the perfusion medium. These effects are similar to those reported for the transport of 5-HT and NA in other cellular systems (Bogdanski, Tissari & Brodie, 1968; Tissari, Schonhofer, Bogdanski & Brodie, 1969; Schultz & Curran, 1972). It has been suggested that the membranal transport of these substances is mediated by a 'carrier' that is dependent on the availability of $Na^+$ for the transport of the solute (Schultz & Curran, 1972). The affinity of the 'carrier' for the solute is increased when $Na^+$ is present in adequate concentrations, but is reduced in its absence. The movement of solute molecules, therefore, depends primarily on the availability of $Na^+$ in the extracellular fluid. Other ions such as $K^+$ and to a lesser extent $Ca^{2+}$ and $Mg^{2+}$ may also be involved in changing the affinity of the carrier for the solute. From these lines of evidence it is tempting to speculate that the (±)-methadone uptake by the lung tissue may involve a similar $Na^+$ dependent/sensitive carrier transport mechanism. Whether a $Na^+$–$K^+$ dependent adenosine-triphosphatase (ATPase) is involved in such a transport system must be determined by further experimentation. It is, however, important to note that the presence of such a $Na^+$–$K^+$ dependent ATPase has been demonstrated in the lung tissue (Mustafa, Ibrahim, Le & Cross, 1969).

It has been shown that the main pathway of metabolism of (±)-methadone involves N-demethylation followed by cyclization (Beckett *et al.*, 1971; Pohland *et al.*, 1971; Sullivan, Smits, Due, Booher & McMahan, 1972). These reactions occur mainly in the liver. The two main metabolites that have been identified are: 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (Beckett *et al.*, 1971; Pohland *et al.*, 1971). These metabolites are also found in the lung tissue after (±)-methadone is given to rats (Sullivan *et al.*, 1972). However, it is not known whether these metabolites are formed in the lung tissue or are transported there after being formed first in the liver. In the present investigation experiments were not conducted to determine quantitatively the extent to which (±)-methadone was metabolized by the lung tissue slices. However, preliminary results obtained with thin-layer chromatographic analysis of incubation medium and tissue homogenate indicate that under the conditions of incubation, (±)-methadone was not metabolized to a measurable degree. Recently Orton *et al.* (1973) have demonstrated that in the rabbit isolated blood-perfused lung preparation, (±)-methadone was metabolized to the extent of 15%. It is, therefore, quite unlikely that the lung slices used in the present study would metabolize much higher proportion of (±)-methadone present in the incubation medium. The fluorescence assay used in the present investigation is specific for (±)-methadone and the two major metabolites mentioned above do not interfer in this assay. Therefore, the T/M ratios calculated are not likely to be very significantly different from the corrected T/M ratios in which allowance for metabolism is made. Actual experiments will have to be conducted to verify this.

One of the more conventional ways of investigating the 'energy' dependency of an active transport process has been to study the effect of a number of chemicals which produce 'metabolic inhibition'. These agents, by a variety of mechanisms, disrupt the cellular metabolic reactions and the attendant 'energy' production and, therefore, adversely and non-specifically affect the active transport processes. Iodoacetate is such a metabolic inhibitor that produces its effect through alkylation of –SH groups of enzymes, particularly those that are involved in the glycolytic pathway (Webb, 1963). Dixon (1937) has shown that triose phosphate dehydrogenase, alpha-glycerophosphate dehydrogenase, and lactate dehydrogenase are inhibited by iodoacetate at concentrations of 10 mM or higher. Other investigators have shown that the inhibition of iodoacetate is relatively slow, not so much because of its rate of penetration, but more so because of the slow rate of alkylation of the enzymes. Fuhrman & Field (1943) have shown that in rat brain slices and cell-free extracts, 0.01 mM iodoacetate produced about 50% inhibition of glycolysis and only 12% inhibition of 'respiration'. This effect was seen at the end of a 3 h incubation period with a latency of about 90 minutes. Only at very high concentrations (5 to 50 mM) was a significant inhibition (>65%) of 'respiration' seen. These results provide a rational explanation as to why in the present study only 20% inhibition of (±)-methadone uptake was seen in 30 min at 1.0 mM iodoacetate concentration. A variable degree of inhibition of the active transport of different compounds (morphine, dihydromorphine, 5-HT, NA, etc.) by iodoacetate has been observed by other investigators (Takemori & Stenwick, 1966; Hug, 1967; Rubin, Owens & Rall, 1968; Iwasawa *et al.,* 1973). One should, therefore, be careful in interpreting results obtained by using iodoacetate as a tool to evaluate the energy requirements or dependency of a transport process.

A mention was made earlier that the inhibitory effect of $Na^+$ lack on (±)-methadone uptake may be related to the involvement of $Na^+-K^+$ dependent ATPase in the transport of (±)-methadone. If this were true, then the inhibitory effect of iodoacetate may also be due to its interaction with the ATPase, since Shamoo & Brodsky (1970) have indicated that $Na^+-K^+$ dependent ATPase is a –SH containing enzyme.

From the foregoing discussion it can be concluded that at low medium concentrations (≤0.5 mM) active transport plays a predominant role in the transport of (±)-methadone. It is also the only demonstrable component at low concentrations because simple diffusion is expected to transport relatively insignificant amounts of (±)-methadone into the lung tissue cells. However, as the (±)-methadone concentration is increased, the active transport becomes saturated and the (±)-methadone uptake becomes concentration-dependent. In other words, at high medium concentrations (M > 1 mM) (±)-methadone is transported by the process of simple diffusion as indicated by the T/M ratio that approaches unity.

It should be noted that at low medium concentrations, when the intracellular concentration of (±)-methadone becomes several times that of the medium concentration it is quite possible that (±)-methadone will diffuse out of the cells into the medium. Thus it becomes difficult to obtain correct estimates of the rate of active transport at any given medium concentration below which the active transport process is not saturated. One way to overcome this difficulty is to analyse the transport of (±)-methadone by a method known as 'system identification' (Sage, 1972). This kind of approach has enjoyed wide attention in the field of engineering. The following is a summary of the analysis of the data, obtained in this investigation by this approach.

It is assumed that the transport of (±)-methadone occurs by two processes: (a) simple diffusion and (b) a saturable active transport process. These two processes can be represented by the equation

$$\frac{dT}{dt} = V + K_D(M - T) \qquad (1)$$

(Akedo & Christensen, 1962) where M is the (±)-methadone concentration in the incubation medium, T is the (±)-methadone concentration in the tissue, and t is the duration of incubation in hours. $K_D(M-T)$ and V are the velocities of the diffusion and of the active transport porcesses respectively. $K_D$ $(M-T)$ is an expression of Fick's law of simple diffusion where $K_D$ is the diffusion constant ($h^{-1}$). $K_D$ is characteristic of the substance being transported and is considered to be independent of t, M and T. V is assumed to be independent of time of incubation (t) as well as of the tissue concentration of (±)-methadone (T). Integration of equation (1) yields:

$$\frac{T}{M} = \frac{V}{K_D}(1 - e^{-K_D t})\frac{1}{M} + (1 - e^{-K_D t}) \qquad (2)$$

In this relationship, $K_D$ and V are the two unknown parameters that can be estimated from experimental data. At very high medium concentration of (±)-methadone, V by definition, becomes independent of M and nearly constant. Thus a T/M vs 1/M plot should result in a straight line. From this plot one can calculate $K_D$ and V (Figure 4). If such a plot is not a straight line but a curve then it could indicate that V is dependent on time over the range of concentration used. After obtaining the value for $K_D$ one can then calculate V at low medium concentrations. Under these circumstances V will vary with M. Analyses of the present data by this procedure were done and the results are summarized below:

(i) $K_D$ was found to be 5.0 $h^{-1}$ when incubations were carried out for 15 and 30 minutes.

(ii) At high medium concentrations of (±)-methadone (1.0–32.0 mM) V became nearly constant ($V_{max}$) and equalled 6.5 μmol $g^{-1}$ $h^{-1}$ (Figure 5). The $V_{max}$ was identical when incubation period was 15 or 30 minutes.

(iii) At all incubation periods and at (±)-methadone concentrations greater than 1.0 mM, V was found to be independent of M. Thus, for 15, 30 and 60 min incubation periods and for medium concentrations (M) from 1.0 to 32.0 mM, calculated values for V (μmol $g^{-1}$ $h^{-1}$, mean ± s.e. mean) were 5.05 ± 0.4, 5.92 ± 0.4 and 6.60 ± 0.5, respectively. At M≤0.05 mM, V was dependent on M, since at these concentrations the 'active transport' process was not saturated. Extensive experimental work at low medium concentrations (M≤0.5 mM), was carried out only for the incubation period of 30 min and, therefore, no definite relationship between the time of incubation (t) and V can be suggested or established.

(iv) At low M, active transport will result in a much higher concentration of (±)-methadone in the tissue (T) than in the medium (M). This was clearly indicated by the fact that at low concentration $T/M \ggg 1$. Hence, the diffusion process will actually transport (±)-methadone out of the tissue and into the medium. This is indicated by the negative sign of diffusion process $[K_D(M-T)]$ at low concentrations of (±)-methadone (Figure 5).

(v) However, at high medium concentrations the active transport process becomes saturated and now (±)-methadone transport becomes concentration-dependent as described by the T/M value which approaches unity. Under these conditions then, simple diffusion process becomes the main transport mechanism. The velocity due to diffusion $[K_D(M-T)]$ became positive (Figure 5), as soon as M became greater than T. Note that the *net* velocity of uptake (dT/dt) by the tissue is positive at all times.

It must be mentioned that the values shown in Figure 5 are calculated values of $[K_D(M-T)]$ and of dT/dt. Therefore, this instantaneous velocity of net transport (dT/dt) at 30 min is not the same as the average net velocity of transport namely $\Delta(T/M)/\Delta t$ obtained at the end of the 30 min incubation period.

This study was supported in part by USPHS Training Grant GM-1217. Authors are grateful to Dr Donald N.H. Chi for his advice in the preparation of this manuscript.

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*(Received July 29, 1975.*
*Revised August 2, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 551–560

# EFFECTS OF *N*-METHYLAMINO ACIDS AND CONVULSANTS ON SPONTANEOUS ACTION POTENTIALS IN GUINEA-PIG CEREBELLAR SLICES

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1 *N*-methyl-$\gamma$-aminobutyrate (*N*-methylGABA), *N*-methylglycine, *N*-methyltaurine and *N*-methyl$\beta$-alanine diminished the frequency of spontaneous spike discharges in guinea-pig cerebellar slices. Usually a weak excitatory effect preceded the inhibition.

2 The inhibitory effects of *N*-methylGABA and *N*-methyl$\beta$-alanine were competitively antagonized by both picrotoxin and strychnine.

3 The inhibitory action of *N*-methyltaurine was competitively suppressed by strychnine and by low concentrations of picrotoxin.

4 The inhibitory action of *N*-methylglycine was suppressed by strychnine but not by picrotoxin. The suppression was competitive at low concentrations of strychnine.

5 *N*-methylDL-glutamate brought about a strong inhibition followed by a strong excitation of the neurones. The inhibitory effects were competitively suppressed by both picrotoxin and strychnine. Neither convulsant affected the excitation.

6 Whereas L- or D-glutamate caused only excitation in the majority of cells examined, a small proportion of the cells exhibited inhibition preceding the excitation by L- or D-glutamate. Such inhibitory effects were suppressed by picrotoxin but not by strychnine.

7 Kinetic analyses of the dose-response curves for the *N*-methylamino acid in the presence or absence of the convulsant indicated that the number of molecules of the amino acid combining with the receptor site to produce a response was 3 for *N*-methylGABA, 2 for *N*-methylglycine, 3 for *N*-methyltaurine, 3 for *N*-methyl$\beta$-alanine. The corresponding value was 1 for *N*-methylDL-glutamate (inhibition). The number of molecules of convulsant combining with the receptor site was calculated to be 2 for picrotoxin with *N*-methylGABA, *N*-methyl$\beta$-alanine and *N*-methylDL-glutamate and 1 for strychnine with all *N*-methylamino acids examined.

## Introduction

We have shown in earlier papers (Okamoto & Quastel, 1973; 1976) that the frequencies of spontaneous action potentials recorded from guinea-pig cerebellar cortex slices are reversibly suppressed by inhibitory amino acids such as $\gamma$-aminobutyrate (GABA), glycine, taurine and $\beta$-alanine. The inhibitory effect of GABA is competitively antagonized by picrotoxin, whereas that of glycine, taurine and $\beta$-alanine is competitively blocked by strychnine. These results indicate that there seem to exist at least two types of receptors for the inhibitory amino acids in the cerebellar cortex, one specific for picrotoxin and the other specific for strychnine.

The present study was carried out to answer the following question: Do the antagonists, picrotoxin and strychnine, still maintain their specificities of action when the amino acid molecule is modified by *N*-alkylation? *N*-methylamino acids were chosen for the present study for the following reasons: (1) Methylation of the *N*-atom does not necessarily result in loss of the physiological activities of the parent amino acids, even though the amino group is considered to combine with the anionic site of the receptor (Curtis & Watkins, 1960). Indeed, there may even be a potentiation of the activity on neuronal excitability. *N*-methylDL-aspartate, for example, is reported to be about five times as potent as DL-aspartate (Curtis & Watkins, 1963). Curtis & Watkins (1960; 1963) and Curtis, Phillis & Watkins (1961) have also described an excitatory effect of *N*-

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methylDL-glutamate, which was as potent as that of DL-glutamate, and inhibitory effects of *N*-methyl$\beta$-alanine, *N*-methylglycine and *N*-methylGABA with cat spinal neurones and isolated spinal cord of the toad, though these were weaker than those of the parent non-methylated amino acids. (2) Some *N*-methylamino acids are known to occur naturally. For example, *N*-methylglycine (sarcosine) exists in the mammalian body (Scott & Labbe, 1974). *N*-methylisoleucine and *N*-methylvaline are present in fungi and *N*-methyltyrosine and *N*-methyltryptophan exist in plants (Guggenheim, 1951). (3) Some *N*-methylamino acids may have physiological importance. For example, the genetic disease, hypersarcosinemia, that is accompanied by neurological disturbance, is associated with the accumulation of *N*-methylglycine in the blood (Scott & Labbe, 1974). *N*-methyl$\beta$-alanine competitively inhibits the active transport of GABA and $\beta$-alanine in cerebral cortex slices, indicating its ability to bind with transport carrier site(s) on neuronal membranes (Johnston & Stephanson, 1976). (4) *N*-methylation of an amine (tryptamine) has been shown to occur with a brain preparation (Boarder, Oon & Rodnight, 1976). It seems possible therefore that *N*-methylation of an amino acid may take place in the brain.

We have found that *N*-methylGABA, *N*-methylglycine, *N*-methyltaurine and *N*-methyl$\beta$-alanine have inhibitory effects similar to those shown by their non-methylated parent amino acids, on the frequencies of spike discharges in guinea-pig cerebellar slices. *N*-methylDL-glutamate exhibits both inhibitory and excitatory actions. Except for the action of *N*-methylglycine, all the inhibitory effects are antagonized by both picrotoxin and strychnine. The effect of *N*-methylglycine is antagonized by strychnine but not picrotoxin.

## Methods

### *Recording of extracellular action potentials*

The preparation of guinea-pig cerebellar cortex slices and the methods used for superfusion and for the recording of action potentials (spikes) have been described in detail previously (Okamoto & Quastel, 1973; 1976). Briefly, after the guinea-pig was killed by stunning, the slice (0.5 mm thick) was prepared from the superior vermis by cutting the isolated cerebellum parallel to its uppermost surface. The slice was then promptly placed on a nylon mesh in the superfusion chamber. All solutions, including the control solution and those containing substances to be tested, were applied to the slice by superfusion (flow rate $\simeq 1$ ml/min) through a glass capillary tip (internal diameter $\simeq 0.3$ mm) placed just above the surface of the slice and close to the recording glass microelectrode (1–2 M$\Omega$), filled with 2.5 M NaCl. The control solution (pH = 7.4) consisted of (mM): NaCl 125, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaH_2PO_4$ 1, $NaHCO_3$ 24 and glucose 11. Amino acids, their sodium salts, or antagonists to be tested for their activities, were dissolved in this solution, the final pH not being affected. The solution was always bubbled with 95% $O_2$ and 5% $CO_2$ in the reservoirs and warmed to 37°C by a water jacket placed near the superfusion tip. Discharge frequencies of extracellular action potentials (spikes/s) were recorded on an oscillographic recorder Model 7402A (Hewlett Packard) and also printed on a Model 2010A printer (John Fluke) after passing through a frequency counter (Digital counter-timer, Model 1952A, John Fluke). In the dose-response curves the spike discharge rates (spikes/s) were obtained by averaging 5 or more values from cells whose discharge rate remained stable for 20 s or more. Amino acid and convulsant-free solutions were applied between the applications of solutions containing amino acid or convulsants to avoid possible desensitiziation due to prolonged exposure of the slice to these agents.

### *Kinetic analyses*

Kinetic analyses of the dose-response relations were carried out in a similar manner to that described in detail in our previous paper (Okamoto & Quastel, 1976). In short, the theoretical dose-response curve, in the absence of an antagonist, was derived from equation (1).

$$p = p_{max}/\{1 + (K/[A])^n\} \qquad (1)$$

where $p$ is the percentage inhibition of spike discharge frequency by the amino acid, K is the concentration of the amino acid (A) giving 50% of the maximum response ($p_{max}$) which is 100, and $n$ is the estimate of the number of molecules of amino acid combining with the receptor site. Values of K and $n$ for each of the dose-response curves were determined from equation (1A), derived from equation (1).

$$K = [A]\{(p_{max}/p) - 1\}^{1/n} \qquad (1A)$$

The observed values of $p$ at various concentrations of A should give a constant value of K at a particular value of $n$ according to equation (1A). This value of $n$ was determined by finding a value, using steps of 0.1 within the range of 0.5 and 4.0, that resulted in constancy of the value of K at different concentrations of A (Okamoto & Quastel, 1976). Values of $n$ and K thus determined agreed with the values obtained by Hill plots, $\log_e[p/(p_{max} - p)]$ *vs* $\log_e[A]$.

It was expected that values of $n$, thus estimated by equation (1A) would be smaller than the real values, when the $n$-value was not unity, because equation (1) was based on the assumption that concentrations of the intermediates of the sequential reactions between the amino acid, A, and the receptor, R, such as AR, . . . $A_{n-1}R$ were negligible compared with those of R and $A_nR$ (see Okamoto & Quastel, 1976).

When a competitive antagonist, I, was present, equation (2) or (3) was applied.

$$p' = p_{max}/\{1+(K/[A])^n[1+([I]/K_I)^m]\} \qquad (2)$$

$$= p_{max}/\{1+(K'/[A])^n\} \qquad (3)$$

where $p'$ is the percentage inhibition of spike discharge frequency in the presence of the antagonist, where K′, the concentration of A to give 50% of $p_{max}$, is equal to $K[1+([I]/K_I)^m]^{1/n}$, and where $K_I$ is the apparent dissociation constant of the antagonist-receptor complex. The value of *m* represents the estimate of the number of antagonist molecules combining with the receptor. The value of *m* was estimated by equation (4).

$$\log_e[(p-p')/p'] = m.\log_e([I]/K_I) - \log_e[p_{max}/(p_{max}-p)] \qquad (4)$$

The slope of the plot of $\log_e[(p-p')/p']$ against $\log_e[I]$ is equal to *m*, with constant [A] (Okamoto & Quastel, 1976). The value of *m* thus estimated, however, should be smaller than the real value when *m*-value is not unity, because equation (2) or (3) was based on the assumption that concentrations of intermediates of the sequential reactions between the antagonist, I, and the receptor, R, such as IR, . . . $I_{m-1}R$ were negligible compared with [R] and $[I_mR]$ (see Okamoto & Quastel, 1976).

The values of $K_I$ were estimated, in view of these considerations, using the nearest higher integers to the values of *n* and *m* obtained from equations (1A) and (4) respectively, by means of the equal-response method (Gaddum, 1943; Hubbard, Llinás & Quastel, 1969) using equation (5) which is derived from equation (1) and (2) when $p = p'$.

$$K_I = [I]/\{([A_1]/[A_2])^n - 1\}^{1/m} \qquad (5)$$

where $[A_1]$ and $[A_2]$ are the concentrations of A giving equal response in the presence and absence of the antagonist respectively.

### *Materials*

*N*-methyl-γ-aminobutyric acid hydrochloride and D-glutamic acid were obtained from Calbiochem., La Jolla, Calif. *N*-methylglycine hydrochloride (Sarcosine), *N*-methyl DL-glutamic acid and picrotoxin were purchased from Sigma Chemical, St. Louis, Mo. *N*-methylβ-alanine was kindly given to us by Dr G.A.R. Johnston, The Australian National University, Canberra. Strychnine sulphate was purchased from Mallinckrodt Chemical, St. Louis, Mo.

The *N*-methylamino acids used were analysed, preparatory to use, by an amino acid analyzer, for contamination with non-methylated parent amino acids. No such contaminants were detected.

Guinea-pigs, male, adult, 350–400 g, were supplied by the Animal Unit, University of British Columbia, Vancouver, B.C., Canada.

## Results

### *Effects of* N*-methylamino acids on spontaneous spike discharge frequencies in cerebellar slices*

*N*-methyl-γ-aminobutyrate (*N*-methylGABA), *N*-methylglycine, *N*-methyltaurine and *N*-methylβ-alanine each diminished the frequency of spontaneous spike discharges in guinea-pig cerebellar slices in a manner similar to that observed with the corresponding non-methylated amino acid (Okamoto & Quastel, 1976). Examples of such inhibitory effects are shown in Figure 1. An early excitation often preceded the inhibition and partial recovery often occurred whilst the amino acid was still present. On removal of the amino acid a transient excitation often preceded the recovery. *N*-methyltaurine, however, seemed to be the exception in that full recovery (Figure 1) rarely occurred.

The time-course of these effects of the *N*-methylamino acids also resembled those of the parent amino acids. For example, the rapid onset of the inhibition due to *N*-methylGABA or *N*-methylβ-alanine was similar to that due to GABA or to β-alanine respectively. The slow onset of the inhibition due to *N*-methyltaurine resembled that due to taurine (Figure 1; Okamoto & Quastel, 1976). The brief inhibition due to *N*-methylglycine followed by full recovery of the discharge rate to the initial value was similar to that observed in the presence of glycine.

The inhibitory effects of the *N*-methylamino acids were observed with all cells tested and were reversible. They were quantitatively related to the concentrations of *N*-methylamino acids in the perfusion medium.

### *Effects of picrotoxin and strychnine on the inhibitory effects of* N*-methylamino acids*

The inhibitory activity of *N*-methylGABA, *N*-methyltaurine or *N*-methylβ-alanine was abolished by both picrotoxin and strychnine (1–50 μM) in all cells tested. The effect of either convulsant was reversible. The action of *N*-methylglycine was antagonized by strychnine (0.2–1 μM) but not by picrotoxin. Examples of these antagonisms by picrotoxin are shown in Figure 1. Similar antagonisms were exhibited by strychnine. The poor specificity of the convulsants on the *N*-methylamino acids is in contrast to their specific effects on the parent, non-methylated, amino acids. For example, the inhibitory action of GABA was abolished by picrotoxin but not by strychnine

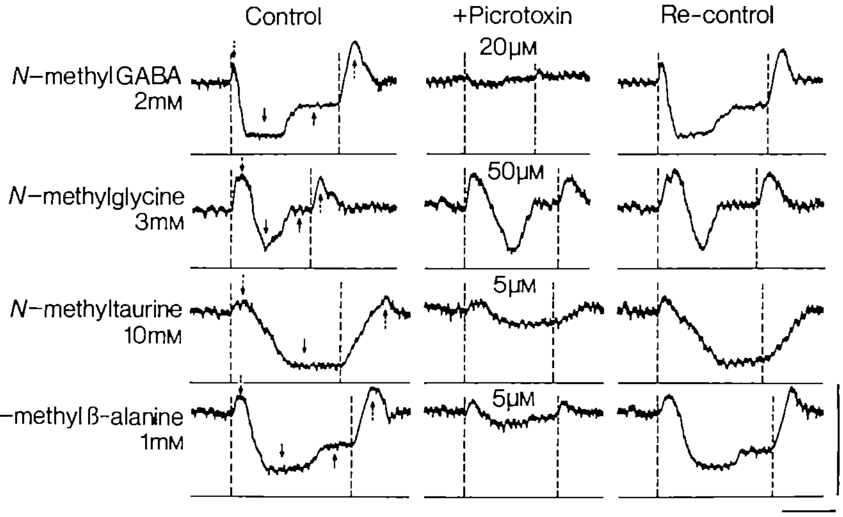

**Figure 1** Typical effects of inhibitory *N*-methylamino acids on spontaneous spike discharge frequencies in guinea-pig cerebellar slices. The antagonistic action of picrotoxin.

Records were obtained from four different cells, one cell being used for each of the *N*-methylamino acids. These were applied, at concentrations shown, for a period given by the interval between the two vertical broken lines. Picrotoxin was applied about 30 s before the application of the *N*-methylamino acid and its effects were shown in the middle records. The left and right records are for the control and re-control (picrotoxin-free) conditions respectively. The latter was recorded about 3 min after the removal of picrotoxin. Scales: vertical = 100 spikes/s; horizontal = 20 s. The solid downward arrows indicate the largest inhibitions, produced by the *N*-methylamino acids, with which the dose-response plots (Figures 2–5) were obtained. The solid upward arrows indicate the partially recovered spike frequencies in the presence of the *N*-methylamino acids. The broken downward arrows and the broken upward arrows indicate the early excitations by the *N*-methylamino acids and the after-excitations on removal of the *N*-methylamino acids respectively. The effects of strychnine (5–50 μM) (not shown) were similar to those of picrotoxin (see Figures 2–5) except for its selective effect on the action of *N*-methylglycine (Figure 3).

whereas that of glycine, taurine or $\beta$-alanine was abolished by strychnine but not by picrotoxin (Okamoto & Quastel, 1976).

### *Dose-response curves of the* N*-methylamino acids*

The effects of *N*-methylGABA, *N*-methylglycine, *N*-methyltaurine and *N*-methyl$\beta$-alanine at different concentrations in the absence or presence of picrotoxin or strychnine are shown in Figures 2–5 where the largest inhibition (as denoted by downward solid arrows in Figure 1) values are recorded. Inhibitions calculated from the partial recoveries given by *N*-methylGABA and *N*-methyl$\beta$-alanine were similar to those given in Figures 2 and 5.

Theoretical log dose-response curves are also given in Figures 2–5. They are derived from equation (1) or (3) in the Methods section. The values of the kinetic constants K and *n* (equation 1) and K′ (equation 3) estimated from the best fitted log dose-response curves, recorded in Figures 2–5, are listed in Table 1.

Examination of the K-values (Table 1) shows that the inhibitory activities of the *N*-methylamino acids were 2–15 times smaller than those of corresponding non-methylated amino acids. The values of K previously obtained with the non-methylated amino acids were 0.12 mM for GABA, 0.10 mM for glycine, 1.0 mM for taurine and 0.35 mM for $\beta$-alanine (see Table 1; Okamoto & Quastel, 1976). The values of *n*, however, i.e., the numbers of molecules of *N*-methylamino acid calculated as combining with the receptor site, were similar to those of the corresponding non-methylated amino acid, i.e., $n = 3$ for *N*-methylGABA; 2 for *N*-methylglycine, and 3 for *N*-methyltaurine. An exception was *N*-methyl$\beta$-alanine whose *n* value was calculated to be 3 whereas that for $\beta$-alanine seemed to be 4 (Okamoto & Quastel, 1976).

*Effects of picrotoxin.* Picrotoxin (1–50 μM) brought about a parallel shift to the right of the dose-response curve due to *N*-methylGABA or *N*-methyl$\beta$-alanine (Figures 2 and 5) indicating an apparent competition (Okamoto & Quastel, 1976) between the *N*-methylamino acid and the convulsant.

Low concentrations (e.g., 0.5 µM) of picrotoxin shifted the dose-response curve of *N*-methyltaurine to the right in parallel, but at high picrotoxin concentrations (>1.0 µM) there was a departure from parallelism, the curve being flattened (Figure 4). It was, therefore, evident that the action of picrotoxin with *N*-methyltaurine was not wholly competitive, the nature of its antagonism varying with its concentration.

The numbers, *m*, of picrotoxin molecules combining with the receptor site, as definined in equation (2) and recorded in Table 1, were estimated from the plots of $\log_e\{(p-p')/p'\}$ against $\log_e[\mathrm{I}]$ (equation 4). These plots, with constant concentrations of *N*-methylGABA, *N*-methyltaurine, and *N*-methylβ-alanine, were linear and the values of *m*, obtained from the slopes, were 2.2 with *N*-methylGABA, 1.7 with *N*-methyltaurine, and 1.4 with *N*-methylβ-alanine (Table 1). However, the value of $m=1.7$ with *N*-methyltaurine, is open to doubt as the effect of picrotoxin with this amino acid was apparently not purely competitive.

It was evident that the value of *m* considerably exceeded unity with *N*-methylGABA and, therefore, that the number of picrotoxin molecules combining with the *N*-methylGABA receptor site was apparently greater than that combining with the GABA receptor site where *m* was 1.0 (Okamoto & Quastel, 1976).

The values of $\mathrm{K_I}$, the apparent dissociation constants of the picrotoxin-receptor complexes, recorded in Table 1, were estimated from the values of the horizontal shifts of the log dose-response curves (Figures 2, 4 and 5) using equation (5).

The value of $\mathrm{K_I}$ for picrotoxin in the presence of *N*-methylGABA ($\mathrm{K_I}=2.5$ µM) was smaller than that found with GABA ($\mathrm{K_I}=5.1$ µM) (Okamoto &

**Table 1** Values of kinetic constants estimated for inhibitory actions of *N*-methylamino acids and for antagonistic effects of convulsants

| | *K or K′ (mM)* | n | *$K_I$ (µM)* | m |
|---|---|---|---|---|
| *N*-methylGABA | 1.32 | 2.4 | — | — |
| + picrotoxin (20 µM) | 4.74 | 2.5 | 2.52 | 2.2 |
| + strychnine (10 µM) | 2.62 | 2.6 | 1.28 | 1.1 |
| *N*-methylglycine | 1.59 | 1.4 | — | — |
| + strychnine (0.2 µM) | 6.00 | 1.5 | 0.055 | 1.7 |
| + strychnine (0.4 µM) | 12.36 | 0.9 | — | — |
| *N*-methyltaurine | 5.45 | 2.4 | — | — |
| + picrotoxin (0.5 µM) | 10.40 | 2.0 | 0.21 | 1.7 |
| + picrotoxin (1 µM) | 17.53 | 1.3 | — | — |
| + strychnine (20 µM) | 18.45 | 2.1 | 0.55 | 1.0 |
| *N*-methylβ-alanine | 0.62 | 2.7 | — | — |
| + picrotoxin (5 µM) | 2.19 | 2.7 | 0.76 | 1.4 |
| + strychnine (10 µM) | 1.10 | 2.0 | 2.18 | 1.1 |
| *N*-methylDL-glutamate | 0.45 | 1.1 | — | — |
| + picrotoxin (3 µM) | 4.94 | 0.9 | 1.12 | 1.9 |
| + strychnine (5 µM) | 1.96 | 0.9 | 1.23 | 1.1 |

The values of K and K′ are concentrations of the *N*-methylamino acids required to give 50% of the maximum inhibition in the absence and presence of the antagonist respectively (equations 1 and 3). The values of *n*, the numbers of molecules of the *N*-methylamino acid combining with the receptor site, calculated from equations 1 and 3, are those that gave the best fits of the theoretical log dose-response curves to the observed values of the percentage inhibitions. The values of $\mathrm{K_I}$ were estimated from the extents of the parallel shifts, at 50% inhibition of the log dose-response curves (Figures 2–5). The values of *n* used for the calculation of $\mathrm{K_I}$ values (using equation 5) were 3, 2, 3, 3 and 1 for *N*-methylGABA, *N*-methylglycine, *N*-methyltaurine, *N*-methylβ-alanine and *N*-methylDL-glutamate respectively. These *n* values are the nearest higher integers to the values of *n* obtained in the absence of the antagonists. The values of *m* shown were estimated using equation (4). The values of *m* used for calculation of $\mathrm{K_I}$ values were the nearest higher integers to the observed *m* values. They were 3 for picrotoxin with *N*-methylGABA, 1 for strychnine with *N*-methylGABA; 2 for strychnine with *N*-methylglycine; 2 for picrotoxin with *N*-methyltaurine, 1 for strychnine with *N*-methyltaurine; 2 for picrotoxin with *N*-methylβ-alanine, 1 for strychnine with *N*-methylβ-alanine, and 2 for picrotoxin with *N*-methylDL-glutamate and 1 for strychnine with *N*-methylDL-glutamate. See Methods section for reasons for employing such whole integers of *n* and *m*.

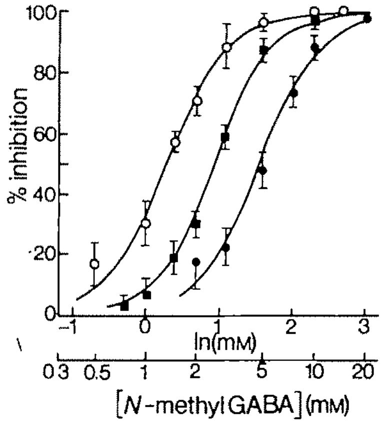

**Figure 2** Log dose-response curves of *N*-methylGABA. Typical effects of picrotoxin and strychnine. Ordinate scale = percentage inhibition of spike discharge frequency by *N*-methylGABA. Abscissa scale = $\log_e$ concentration (mM) of *N*-methylGABA. The values plotted were obtained from the largest inhibitions, as denoted by solid downward arrows in Figure 1. They are the means of values found with 5–14 cells in several slices. Vertical lines show s.e. means. (○) Control; (●) with 20 µM picrotoxin; (■) with 10 µM strychnine. Lower concentrations (down to 1 µM) of both of the convulsants were also tested. They gave similar but smaller parallel shifts. The lines are theoretical curves (equation 1 or 3) that gave the best fits to the observed values. Values of *n*, K and K′ are given in Table 1.

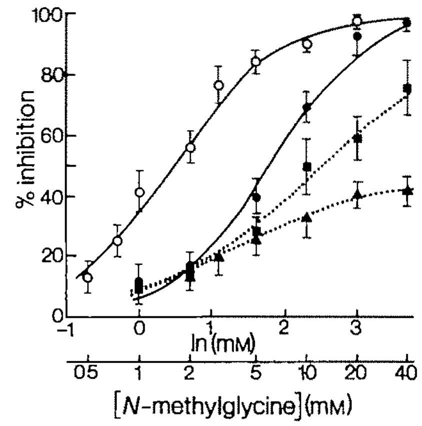

**Figure 3** Log dose-response curves for *N*-methylglycine. Typical effects of strychnine. Ordinate scale = percentage inhibition of spike discharge frequency by *N*-methylglycine. Abscissa scale = $\log_e$ concentrations (mM) of *N*-methylglycine. The values plotted were obtained from the largest inhibitions (Figure 1, solid downward arrows). They are the means of values found with 5–12 cells in several slices. Vertical lines show s.e. means. (○) Control; (●) with 0.2 µM strychnine; (■) with 0.4 µM strychnine; (▲) with 1 µM strychnine. The solid lines are theoretical curves (equation 1 or 3) that gave the best fits to the observed values. The dotted lines are not theoretical curves, they simply connect the observed values. Values of *n*, K or K′ are given in Table 1. Picrotoxin (1–50 µM) was without effect on the inhibitions by *N*-methylglycine.

Quastel, 1976). Judging from the $K_I$ values (Table 1), picrotoxin blocked the actions of *N*-methyltaurine and *N*-methylβ-alanine more effectively than those of *N*-methylGABA or GABA.

*Effects of strychnine.* Strychnine (1–50 µM) abolished the inhibitory effects of all the *N*-methylamino acids tested in all cells examined. Its action was reversible.

The presence of this convulsant caused a parallel shift to the right of the log dose-response curves of *N*-methylGABA, *N*-methyltaurine and *N*-methylβ-alanine (Figures 2, 4 and 5).

The log dose-response curve of *N*-methylglycine was shifted to the right in a parallel manner when the strychnine concentration was 0.2 µM (Figure 3). At higher concentrations (0.4 µM and 1 µM) of strychnine the curves departed from parallelism and became flatter (Figure 3). It seemed, therefore, that the strychnine antagonisms of *N*-methylGABA, *N*-methyltaurine and *N*-methylβ-alanine were competitive, whilst that of *N*-methylglycine was not competitive at relatively high strychnine concentrations.

The *m* values for strychnine (Table 1), estimated from equation (4), were 1.1 with *N*-methylGABA or *N*-methylβ-alanine, 1.0 with *N*-methyltaurine and 1.7 with *N*-methylglycine. The numbers, therefore, of strychnine molecules combining with the receptor site(s) to abolish the inhibitory actions of the *N*-methylamino acids, seemed to be 1 with the exception of *N*-methylglycine, whose *m*-value, as with that for picrotoxin and *N*-methyltaurine, is open to doubt.

The *m* value for strychnine with glycine, taurine or β-alanine has been previously estimated as 1 (Okamoto & Quastel, 1976).

The $K_I$ values (dissociation constant) for strychnine (estimated similarly to those for picrotoxin) are given in Table 1. A comparison of these values with those found in presence of glycine ($K_I$=0.25 µM), taurine ($K_I$=0.35 µM) or β-alanine ($K_I$=0.48 µM) (Okamoto & Quastel, 1976) shows that strychnine was as effective on *N*-methyltaurine as on taurine, less effective on *N*-methylβ-alanine than on β-alanine and

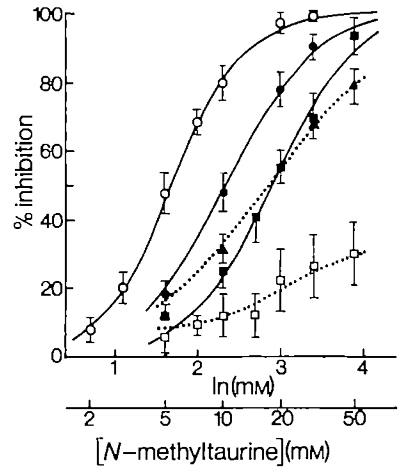

**Figure 4** Log dose-response curves for *N*-methyltaurine. The effects of picrotoxin and strychnine. Ordinate scale = percentage inhibition of spike discharge frequency by *N*-methyltaurine. Abscissa scale = $\log_e$ concentration (mM) of *N*-methyltaurine. The values plotted were obtained from the largest inhibitions (Figure 1, solid downward arrows). They are the means of values found with 6–21 cells in several slices. Vertical lines show s.e. means. (○) Control; (●) with 0.5 μM picrotoxin; (▲) with 1 μM picrotoxin; (□) with 5 μM picrotoxin; (■) with 20 μM strychnine. Lower concentrations (down to 1 μM) of strychnine were also tested. They gave similar but smaller parallel shifts. The solid lines are theoretical curves (equation 1 or 3) that gave the best fits to the observed values. The dotted lines are not theoretical curves; they simply connect the observed values. Values of *n*, K or K′ are given in Table 1.

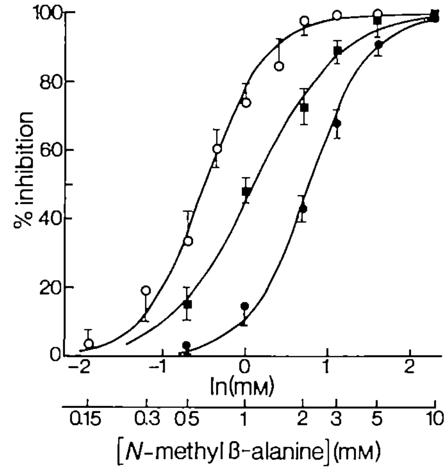

**Figure 5** Log dose-response curves for *N*-methyl*β*-alanine. The effects of picrotoxin and strychnine. Ordinate scale = percentage inhibition of spike discharge frequency by *N*-methyl*β*-alanine. Abscissa scale = $\log_e$ concentration (mM) of *N*-methyl*β*-alanine. The values plotted were obtained from the largest inhibitions (Figure 1, solid downward arrows). They are the means of values found with 8–15 cells in several slices. Vertical lines show s.e. means. (○) Control; (●) with 5 μM picrotoxin; (■) with 10 μM strychnine. Strychnine or picrotoxin (1–50 μM) gave similar parallel shifts but they varied in extent according to the concentrations. The lines are theoretical curves (equation 1 or 3) that gave the best fits to the observed values. Values of *n*, K or K′ are given in Table 1.

probably more effective on *N*-methylglycine than on glycine.

### *Effects of* N-*methyl*DL-*glutamate and* L- *or* D-*glutamate on spontaneous spike discharge frequencies*

*Action of* N-*methyl*DL-*glutamate.* Application of *N*-methylDL-glutamate resulted in a consistent biphasic action in all (54) cells investigated. There was always a prompt initial inhibition followed by a strong excitation (Figure 6). The inhibitory and excitatory activities of this amino acid were reversible and they increased according to the concentration. A small early excitation (Figure 6) often preceded the inhibition. With 2 cells out of 54 cells examined the strong excitation preceded the inhibition. At relatively low concentrations (<0.5 mM) of *N*-methylDL-glutamate its action was predominantly inhibitory.

*Action of* L- *or* D-*glutamate.* Application of L-glutamate occasionally brought about a biphasic action similar to that given by *N*-methylDL-glutamate. Typical results are shown in Figure 6. Such biphasic action was only observed on about 8 of more than 100 cells examined.

D-Glutamate behaved in a manner similar to that of L-glutamate, but its potency (inhibitory and excitatory) was much weaker than that of L-glutamate (results not shown).

### *Effects of convulsants on the inhibitory activity of* N-*methyl*DL-*glutamate*

Whereas the occasional inhibitory effects of L- and D-glutamates were abolished by picrotoxin (5–50 μM) and not by strychnine (5–50 μM), the persistent effects of *N*-methylDL-glutamate were antagonized by both picrotoxin (1–50 μM) and strychnine (1–50 μM) (Figure 6). The abolition of the inhibitions by the convulsants resulted in an earlier onset of the excitation whose magnitude was unaffected (Figure 6). The block of the inhibitions by the convulsants was always reversible with *N*-methylDL-glutamate but not

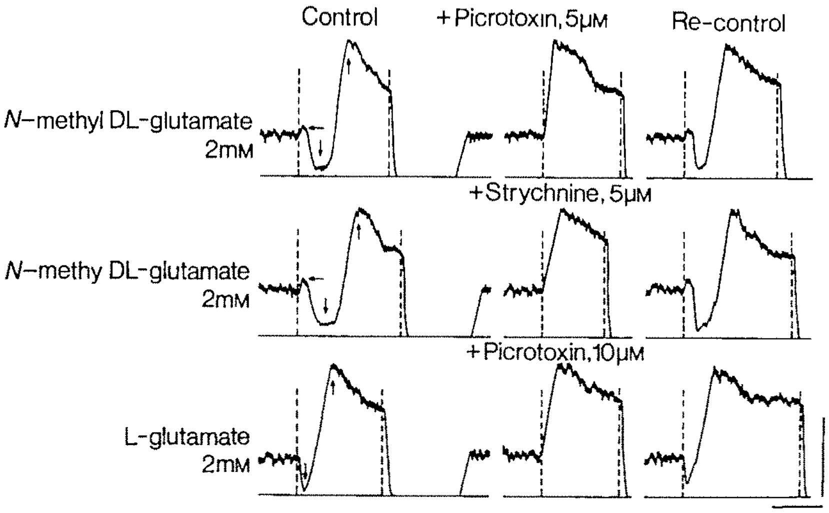

**Figure 6** Typical effects of *N*-methylDL-glutamate and of L-glutamate on spike discharge frequencies. The antagonistic actions of picrotoxin and strychnine. Records were obtained from three different cells, one cell being used for each set of results. *N*-methylDL-glutamate or L-glutamate was applied, at the concentrations shown, for a period given by the interval between the two vertical broken lines. Picrotoxin or strychnine was applied about 30 s before the application of the *N*-methylDL-glutamate or L-glutamate. Their effects are shown in the middle records. The left and right records are for the control and re-control (the convulsant-free) conditions respectively. The latter was recorded about 5 min after the removal of the convulsants. Scales: vertical = 100 spikes/s; horizontal = 20 seconds. The downward arrows and upward arrows indicate respectively the inhibition and excitation by *N*-methylDL-glutamate or L-glutamate. Horizontal arrows indicate the early excitations. D-Glutamate, not shown, gave inhibitory and excitatory effects similar to those of L-glutamate. Its inhibitory effect was blocked by picrotoxin (10 μM). Strychnine at concentrations up to 50 μM did not affect the inhibitory action of L- or D-glutamate. Silent periods occurred immediately after the removal of *N*-methylDL-glutamate or L-glutamate. This is demonstrated in the control column.

always so with L- or D-glutamate. Repeated applications of picrotoxin caused the inhibitory action of L- and D-glutamate to become smaller.

### *Dose-response curves of inhibitory effect of* N-*methylDL-glutamate*

The log dose-response curves of the inhibitory effect of *N*-methylDL-glutamate and of the antagonisms exerted by picrotoxin and strychnine, are shown in Figure 7.

The value of *n* giving the best fit of the theoretical dose-response curve to the experimental values was 1.1 and the value of K was estimated to be 0.45 mM (Table 1). It seemed, therefore, that only one molecule of *N*-methylDL-glutamate was necessary to combine with its receptor site to bring about inhibition of frequency of the spike discharges.

The application of picrotoxin (1–50 μM) or strychnine (1–50 μM) brought about parallel shifts of the log dose-response curves to the right and the values of *n* were unchanged ($n = 0.9$, Table 1). These results indicated competitive antagonism by these convulsants.

The number of molecules of picrotoxin combining with the receptor site to yield antagonism was estimated to be 1.9 and that for strychnine was estimated to be 1.1 (Table 1). It would seem that two molecules of picrotoxin per receptor site were required for the *N*-methylDL-glutamate antagonism, as shown also for *N*-methylGABA and *N*-methyl*β*-alanine (Table 1). Only one molecule of strychnine seemed to be necessary for the antagonism, as shown also for *N*-methylGABA, *N*-methyltaurine and *N*-methyl*β*-alanine (Table 1).

The values of $K_I$ were estimated to be 1.1 μM for picrotoxin and 1.2 μM for strychnine in the presence of *N*-methylDL-glutamate (Table 1).

## Discussion

The present study has demonstrated the inhibitory effects, on the frequencies of spontaneous spike discharges displayed by guinea-pig cerebellar slices, of *N*-methylGABA, *N*-methyltaurine, *N*-methylglycine, *N*-methyl*β*-alanine and *N*-methylDL-glutamate as well as the excitatory effect of *N*-methylDL-glutamate.

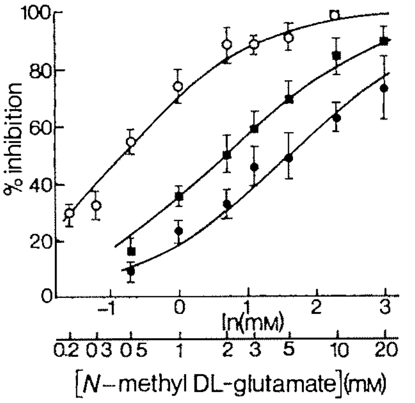

**Figure 7** Log dose-response curves for the inhibitory actions of *N*-methylDL-glutamate. The effects of picrotoxin and strychnine. Ordinate scale = percentage inhibitions of spike discharge frequency by *N*-methylDL-glutamate. Abscissa scale = $\log_e$ concentration (mM) of *N*-methylDL-glutamate. The values plotted were obtained from the largest inhibitions, as denoted by downward arrows in Figure 6. They are the means of values found with 5–13 cells in several slices. Vertical lines show s.e. means. (○) Control; (●) with 3 µM picrotoxin; (■) with 5 µM strychnine. Similar parallel but variable shifts of the control curve were observed at concentrations of picrotoxin between 1 and 20 µM. The lines are theoretical curves (equation 1 or 3) that gave the best fits to the observed values. Values of *n*, K or K′ are given in Table 1.

These activities of *N*-methylamino acids were not due to the presence in the preparations of these amino acids and in superfusing media, of the non-methylated amino acids for the following reasons: (1) No such contaminating amino acids were detected by amino acid analysis, (2) their inhibitory effects were antagonized by both picrotoxin and strychnine in a manner different from that exerted with the non-methylated amino acids, (3) kinetic analyses revealed different affinities for their receptor sites from those shown by the corresponding non-methylated amino acids.

It is particularly noteworthy that the inhibitory activities of *N*-methylGABA, *N*-methyltaurine, *N*-methyl$\beta$-alanine and *N*-methylDL-glutamate were antagonized by both picrotoxin and strychnine, a phenomenon distinct from the specific effects in cerebellar slices of picrotoxin on inhibitions due to GABA and of strychnine on those due to glycine, taurine and $\beta$-alanine.

The question at once arose as to whether the *N*-methylamino acids (apart from *N*-methylglycine) are able to combine with both the receptor group selectively specific for picrotoxin and that (or those) specific for strychnine. It would be unlikely, if such combinations occur independently, that one antagonist would bring about almost complete abolition of the inhibitory activity of the *N*-methylamino acid. For example, the inhibitory effects of *N*-methylGABA and *N*-methyl$\beta$-alanine at low concentrations are almost completely abolished by either picrotoxin or strychnine (see lower portions of the dose-response curves in Figures 2 and 5). It is perhaps possible that *N*-methylamino acids must combine with both picrotoxin-sensitive and strychnine-sensitive receptors, before an inhibitory action on the spike frequency can occur. In this case, blockade of either of the receptors by either strychnine or picrotoxin, would abolish the inhibitory activity of the *N*-methylamino acid. Another possibility is the existence of a special type of receptor site, for the *N*-methylamino acids, that is affected by both picrotoxin and strychnine. Presumably a receptor originally sensitive to only one convulsant could become sensitive to the other convulsant after combination with a *N*-methylamino acid. Clearly further studies are required to distinguish between these and other possible explanations.

The inhibitory activity of *N*-methylglycine is abolished only by strychnine and not by picrotoxin. There is no need, therefore, to consider, a combined picrotoxin- and strychnine-sensitive receptor site.

The parallel shifts of the log dose-response curves of *N*-methylGABA, *N*-methyl$\beta$-alanine and *N*-methylDL-glutamate brought about by the convulsants show the antagonism to be of a competitive nature (Okamoto & Quastel, 1976). High concentrations (such as 50 µM) of the convulsants were not tested because of the possible occurrence of non-specific actions of the convulsants at such high concentrations (Freeman, 1973).

The antagonism by strychnine of the action of *N*-methylglycine, however, appeared to be competitive only at the low concentration (0.2 µM) of strychnine. At higher concentrations (0.4 or 1 µM) it was not competitive (Figure 3). *N*-methyltaurine also seems to be antagonized competitively by only a low concentration (e.g. 0.5 µM) of picrotoxin (Figure 4).

### *Biphasic action of* N*-methyl*DL*-glutamate*

*Inhibitory effects.* As the occasional initial inhibitory effects of both L- and D-glutamate (Figure 6) on the spike discharges were blocked by picrotoxin and not by strychnine (Figure 6), the release of endogenous GABA, or closely related substances, from nerve terminals of interneurones such as the basket or stellate cells that are tonically inhibitory to the Purkinje cells, might be responsible for the initial inhibitory effects. This, however, is not the explanation of the inhibitory action of *N*-methylDL-glutamate, because the action is antagonized by both picrotoxin and strychnine (Figures 6 and 7).

*Excitatory effects.* The excitatory action of *N*-methylDL-glutamate resembled that of L- or D-glutamate. However, the maximum increases of excitation achieved by these amino acids were 600% by L-glutamate, 900% by *N*-methylDL-glutamate and 450% by D-glutamate. Neither picrotoxin nor strychnine affected the excitation.

We gratefully acknowledge financial assistance of the Medical Research Council of Canada.

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*(Received June 16, 1976.*
*Revised November 1, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 561–563

# CARDIOVASCULAR EFFECTS OF $\Delta^9$-TETRAHYDROCANNABINOL IN CONSCIOUS AND ANAESTHETIZED DOGS

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1 Temporal effects of $\Delta^9$-tetrahydrocannabinol (THC) on heart rate and blood pressure in conscious dogs were compared to those in anaesthetized dogs.

3 In conscious dogs, THC in doses of 0.25 and 0.1 mg/kg resulted in maximal heart rate reductions of 48 and 41%, respectively, and in no significant change in blood pressure.

3 In anaesthetized animals THC in doses of 0.5 and 0.25 mg/kg caused a peak reduction in heart rate of 38 and 34%, and of blood pressure of 24 and 8%, respectively.

4 The results demonstrate that the bradycardia in response to THC in dogs is independent of the concomitant anaesthesia.

5 We conclude that the discrepancy between heart rate response to THC in dogs and in man is due to a species difference.

## Introduction

Whereas $\Delta^9$-tetrahydrocannabinol (THC) consistently causes tachycardia in man regardless of the route of administration (Hollister, Richards & Gillespie, 1968; Johnson & Domino, 1971; Galanter, Wyatt, Lemberg, Weingartner, Vaughan & Roth, 1972), it is well documented that it causes bradycardia when injected intravenously in anaesthetized dogs (Dewey, Harris, Howers, Kennedy, Granchelli, Pars & Razoan, 1970; Cavero, Kubena, Dziak, Buckley & Jandhyala, 1972; Cavero, Solomon, Buckley & Jandhyala, 1973). Information concerning the cardiovascular effects of THC in conscious dogs is sparse (Dewey *et al.*, 1972; Lahiri, Ladder & Hardman, 1972). The purpose of this study was, therefore, to investigate more fully, in conscious animals, the cardiovascular effects of THC in order to establish whether the discrepant effects of THC in man and dog are due to a species difference or result from the anaesthesia. Such information will clarify the extent to which mechanisms of THC effects in man can be deduced from experiments with dogs.

## Methods

### *Conscious dogs*

Three mongrel dogs weighing $12.2 \pm 0.6$ kg were surgically prepared with exteriorized carotid arteries in skin loops (Gershon & Lang, 1962) for continuous measurements of arterial blood pressure and heart rate. One dog without surgical preparation and weighing 13.5 kg was used for heart rate recording. All dogs were trained to stand in a Pavlov-type harness and accustomed to experimental procedures. Needle-type ECG leads were used for recording Lead II. Heart rate was determined by counting beats/min at the designated times. Control blood pressure and heart rate were measured 20–40 min after the injection of 0.2 ml of ethanol, and were identical to the control values obtained before ethanol. These control measurements were immediately followed by an intravenous injection of THC (0.1 or 0.25 mg/kg) dissolved in the same volume of ethanol. Behaviour was observed before and for 70 min following drug administration. In a single animal the two doses of THC were administered 2–3 weeks apart in random order.

### *Anaesthetized dogs*

Male mongrel dogs weighing $17.3 \pm 0.2$ kg were lightly anaesthetized with intravenously administered sodium pentobarbitone (25 mg/kg). Rectal temperature was maintained at $38 \pm 0.5$°C. A femoral venous catheter was used for drug injection and a femoral arterial catheter for measurements of arterial pressure and

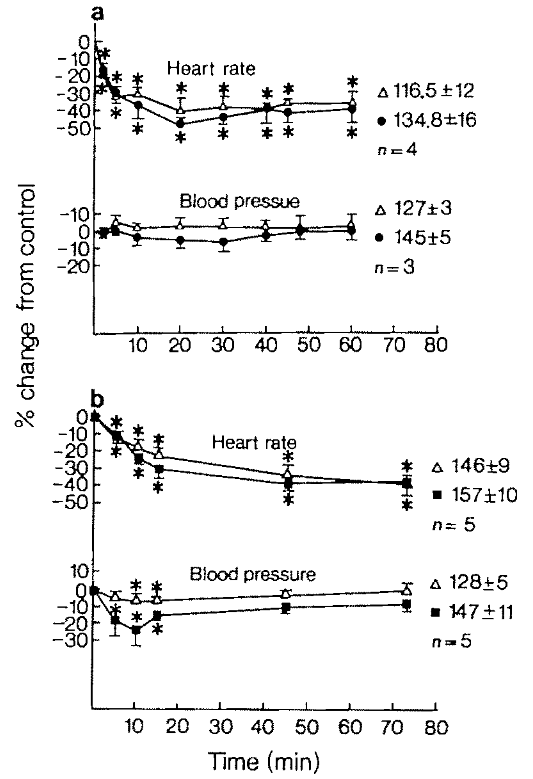

**Figure 1** Temporal effects of intravenous administration (a) of 0.25 (●) and 0.10 mg/kg (△) $\Delta^9$-tetrahydrocannabinol in awake dogs, and (b) of 0.50 (■) and 0.25 mg/kg (△) $\Delta^9$-tetrahydrocannabinol in anaesthetized dogs, on heart rate and mean arterial blood pressure. Percentage changes from control are indicated on the ordinate scale. Each point and vertical line represents the mean ± s.e. mean.

* Significant change from control ($P<0.05$ by Student's $t$ test).

Mean control values ± s.e. mean are shown on the right-hand side for each parameter.

heart rate. Needle-type ECG leads were used for recording Lead II. THC (0.25 and 0.5 mg/kg) and cannabidiol (CBD, 0.5 mg/kg) were dissolved in ethanol (0.25 ml) for injection.

## Results

### *Conscious dogs*

Control values of heart rate and mean arterial pressure, and the effect of THC (0.25 and 0.10 mg/kg) on these two functions in conscious dogs are depicted in Figure 1. A precipitous fall in heart rate occurred during the initial 5 min (30% of control) following both doses of THC. Heart rate reached minimum (41–48% of control) 20 min after THC injection and remained at that level for 60 minutes. The degree of bradycardia in response to THC was not dose-related. Bradycardia was associated with sinus arrhythmia in both conscious and anaesthetized dogs. Mean arterial blood pressure in response to THC was not different from control.

Rear leg ataxis, front leg rigidity and sagging back appeared as early as 5 min after THC injection, and was dose-related. Hyper-reactivity to touch and sound occurred approximately 10 min after drug injection, and lasted throughout the experiment. Spontaneous jerks and intermittent head-drops ('nodding') were clearly observed 5 to 40 min after drug injection in 6 out of 8 experiments performed. Mydriasis, conjunctival injection and leg tremor were also observed.

### *Anaesthetized dogs*

The effects of 0.5 and 0.25 mg/kg THC on heart rate and mean arterial blood pressure, calculated in terms of percentage change from control, are summarized in Figure 1. Heart rate diminished rapidly during the first 15 min and was at a minimum 45 and 75 min after both doses of THC. No statistically significant difference between the two doses was observed.

Hypotension was mild and reached its peak 10 min after drug injection. This effect was dose-related, in that blood pressure decreased by 8% with 0.25 mg/kg and by 24% with 0.5 mg/kg of THC.

An intravenous injection of cannabidiol (0.5 mg/kg) in 3 dogs resulted in no alteration in heart rate or in mean arterial blood pressure during 45 min of observation.

## Discussion

The significance of the present study lies in the fact that the cardiovascular effects of THC were compared in both conscious and anaesthetized animals. Moreover, the cardiovascular measurements in conscious animals were made intra-arterially, thus minimizing the effect of stress inherent in other measurement techniques (Dewey *et al.*, 1972). The doses employed in our conscious dog experiments were within the range of those used by marijuana smokers.

The effect of THC on blood pressure in human subjects is variable. Our findings that 0.5 mg/kg THC induces a transient hypotension in anaesthetized dogs, and 0.1 and 0.25 mg/kg of THC produce no blood pressure changes in conscious dogs, confirm previous studies in anaesthetized dogs (Cavero *et al.*, 1973) and extend those in conscious dogs in which much higher doses of THC were employed (Dewey *et al.*, 1972;

Lahiri *et al.*, 1972). The discrepancy in blood pressure responses between conscious and anaesthetized animals might be attributed to the lack of compensatory vascular reflexes in the anaesthetized preparation.

Previous results on the effects of THC on heart rate in anaesthetized animals (Dewey *et al.*, 1970; Cavero *et al.*, 1972) stand in contrast to those in human subjects (Perez-Reyes, Timmons, Lipton, Davies & Wall, 1972; Galanter *et al.*, 1972; Johnson & Domino, 1972), and could have been attributed in part to an interaction of THC with anaesthetics. The present finding of bradycardia in both anaesthetized and conscious dogs dispels this notion. This difference may stem, at least in part, from the marijuana-induced 'high' in human subjects.

While this possibility is supported by the finding that human subjects developed both psychological tolerance as well as bradycardia in response to chronic THC (Benowitz & Jones, 1975), the lack of temporal correlation between the euphoria and bradycardia (Gallanter *et al.*, 1972) makes this explanation less likely. It is, therefore, concluded that the discrepancy in heart rate response to THC in man and dog is due to a species difference. Furthermore, one should exercise caution in deducing that the cardiovascular mechanism involved in THC effects in experiments on dogs apply to man.

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*(Received July 12, 1976.*
*Revised September 28, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 565–569

# THE INFLUENCE OF GASTRIC EMPTYING ON PLASMA CONCENTRATIONS OF THE ANALGESIC, MEPTAZINOL

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1 The influence of gastric emptying on the rate of absorption and peak plasma concentrations of meptazinol was studied in monkeys by comparison of the plasma concentrations of the drug achieved after intragastric and intraduodenal administration of the compound. Absorption was much more rapid and plasma concentrations were very much higher after intraduodenal than after intragastric dosage.

2 Determination of the 'normal' gastric emptying time in rats and monkeys showed that this was much longer in the monkey than in the rat. In addition, meptazinol was shown to have a pronounced retarding action on gastric emptying in the monkey, an effect which was not apparent in the rat.

3 The significance of these observations in relation to the species difference in oral potency of the drug is discussed.

## Introduction

Meptazinol [*m*-(3-ethyl-1-methyl hexahydro-1H azepin-3-yl)phenol] hydrochloride is a potent new analgesic agent with an apparently low addiction liability. When given subcutaneously, the compound has been shown to be equipotent with pentazocine in both the rat tail flick and mouse acetylcholine writhing tests (Goode & White, 1971). In the monkey leg shock test the compound was almost 2.5 times more potent than pentazocine after intramuscular administration.

Although meptazinol was shown to have good analgesic activity after parenteral dosage, it was found to be considerably less potent after oral administration. For example, in the rat tail flick test the $ED_{50}$ after oral dosage was 32 mg/kg compared to 8.7 mg/kg after subcutaneous administration (Goode, unpublished). In the monkey this difference was even more pronounced. After intramuscular injection the $ED_{50}$ was 6.0 mg/kg but oral doses up to 10 times this failed to elicit a significant increase in shock threshold (Malis, unpublished).

This lower oral potency was reflected in lower plasma and tissue concentrations of the drug after oral compared with patenteral administration and was particularly marked in the monkey (Franklin & Aldridge, 1976). However these lower drug concentrations were not due to poor absorption since in both species over 70% of the radioactivity from an oral dose of the labelled compound was recovered in the urine.

Studies on the biotransformation of the compound indicated that it was extensively metabolized to the glucuronide conjugate in both species. However, a comparison of rat and monkey hepatic glucuronyl transferase showed that the enzyme activity in monkey liver was no greater than that in the rat (Franklin, 1975).

Closer inspection of the absorption data in the two species showed that the compound was more slowly absorbed in monkeys than in rats. This may have resulted in a flattening of the plasma concentration/time profile and in more extensive metabolism of the drug.

A major determinant of the rate of absorption of weakly basic drugs may be the rate of gastric emptying, since this determines the speed of passage of the drug to the site of absorption in the small intestine. This paper describes studies on the influence of gastric emptying on the rate of absorption of meptazinol. The consequent effect of this change in absorption rate on the magnitude of the plasma concentration is also described.

## Methods

### *Radiochemicals*

The random tritiation of meptazinol was carried out at the Radiochemical Centre, Amersham, Bucks. Labile tritium was removed by repeated dissolution in aqueous methanol and subsequent evaporation under reduced pressure. The further purification of the compound was undertaken in these laboratories. The biological stability of the tritium label was demonstrated by the lack of significant quantities of tritiated water in the urine of animals dosed with the material. Radiochemical chemical purity was greater than 95% as shown by thin layer chromatography and the preparation had a specific activity of approximately 70 $\mu$Ci/mg.

[$^{14}$C]-Polyethylene glycol (PEG) 4000 of specific activity 21.4 $\mu$Ci/mg was used as an unabsorbable marker to determine the half-life of gastric emptying. This was obtained from the Radiochemical Centre, Amersham, Bucks.

### *Animals*

Male rats, CD strain, descended from Sprague-Dawley were supplied by Charles River (U.K.) Ltd, Manston, Kent. They were maintained on a diet of Oxoid 41B pellets and weighed 250–350 g at the time of use.

Male Red Patas monkeys (*Erythrocebus patas*) weighing 3–5 kg were obtained from Shamrock Farms Ltd, Brighton, Sussex. They were maintained on an Oxoid 41B diet supplemented by fresh fruit.

### *Administration of meptazinol and collection of blood samples*

Monkeys were fasted overnight before drug administation but were allowed free access to water. Food was withheld for 2 h after dosage. The drug was administered orally by gastric intubation and intraduodenally by injection through a rubber septum attached to the proximal end of a duodenal cannula. In both cases the drug was given as a solution of the hydrochloride salt in isotonic saline. The dose was 25 mg/kg and the vehicle volume 5 ml/kg.

At various times up to 7 h dosage, samples (2 ml) were taken from a femoral vein and immediately transferred to heparinized containers.

### *Measurement of radioactivity*

Radioactivity was determined by liquid scintillation counting in a Packard Tricarb spectrometer, model 3380, equipped with automatic external standardization. The method of sample preparation and the scintillants employed were the same as those previously described by Franklin & Aldridge (1976).

### *Permanent cannulation of the monkey duodenum*

This was performed by Dr B. Alps of the Pharmacology Dept., Wyeth Institute of Medical Research. Two female Patas monkeys were anaesthetized with halothane and a midline abdominal incision made to expose the gastrointenstinal tract. Taking care to minimize disruption of the vasculature, one end of a small plastic cannula (Portex manometer line type 200/490/100) was inserted through a small incision made in the duodenum. This was secured by a circular purse string suture and then anchored by stitching to the lower region of the stomach. The other end of the cannula was exteriorized through the peritoneum, capped with a small rubber septum and pushed beneath a fold of skin near the animals groin. The main incision was closed and the animal allowed to recover. The implantation of the cannula did not appear to affect the animals in any obvious way. Food consumption, body weight and general behaviour seemed normal. There was no evidence of altered gastro-intestinal motility since the animals did not suffer from constipation or diarrhoea.

### *Measurement of gastric emptying*

The rate of gastric emptying was determined by measurement of the disappearance of [$^{14}$C]-PEG 4000, from the stomach. The utility of this material as an unabsorbable marker to measure gastrointestinal motility was described by Miller & Schedl (1970).

*Rats.* Groups of eight male rats were fasted overnight and 1 h before dosing, access to water was withdrawn. Each animal was given, by gastric intubation, 3 ml of isotonic saline containing 1 $\mu$Ci [$^{14}$C]-PEG 4000. Groups were killed at 10, 20 and 30 min after dosing, by injection of ethanol directly into the brain (Pfeiffer & Muller, 1967). The abdomen of each animal was opened and the stomach clamped at either end as quickly as possible after death. The stomach was then removed and the contents were washed out with saline, and aliquots subjected to liquid scintillation counting. Since gastric emptying of a liquid appears to be a first order process the speed of emptying could be conveniently expressed in terms of a half-life, determined from a plot of $\log_{10}$ dose remaining in stomach against time.

After establishing the half-life of gastric emptying under these conditions, the influence of meptazinol on the process was investigated. Thus, unlabelled meptazinol (8 mg$\equiv$dose of 25 mg/kg) was added to each 3 ml portion of isotonic saline containing the [$^{14}$C]-PEG 4000 and the half-life of gastric emptying re-determined.

*Monkeys.* A group of four male monkeys were fasted overnight. The following morning each animal was given, by gastric intubation, 15 ml isotonic saline, containing 5 $\mu$Ci [$^{14}$C]-PEG 4000. Thirty minutes later the animals were restrained and their stomachs washed out with 3 x 50 ml isotonic saline. The amount of radiolabelled marker present was subsequently determined by liquid scintillation counting. Assuming disappearance from the stomach to proceed in an exponential fashion, the half-life of emptying was calculated from the expression

$$T_{\frac{1}{2}} = \frac{0.301 \times t}{\log_{10} \frac{(x_0)}{(x_t)}}$$

where t = time after dosing (in this case 30 min); $x_0$ = % remaining in stomach at time zero (=100%); $x_t$ = % remaining in stomach at time t.

After establishing the half-life gastric emptying under these conditions, the influence of meptazinol on the process was investigated. Thus, unlabelled meptazinol (75 mg ≡ dose of 25 mg/kg) was added to each 15 ml portion of isotonic saline containing the [$^{14}$C]-PEG 4000 and the half-life of gastric emptying re-determined.

## Results

### *Comparison of plasma concentrations of meptazinol achieved after intragastric or intraduodenal dosage of the drug to monkeys*

Intraduodenal dosage of meptazinol resulted in very much more rapid absorption of the drug than occurred after intragastric dosing (Table 1). Peak plasma concentrations of the unchanged drug were achieved after only 15–30 min compared to 3.5 h after oral administration and were at least five times higher than when the same dose was given intragastrically (Table 1). Peak plasma concentrations of free meptazinol after intraduodenal administration ranged from 300 to 470 ng/ml compared to only 9 to 67 ng/ml after the same dose was given orally. Elimination of the drug from the plasma of the intraduodenally dosed animals proceeded with a half-life of 1.18 hours. In the orally dosed animals elimination was also rapid but the plasma concentrations were too low to enable accurate calculation of the half-life.

### *Effect of meptazinol on gastric emptying time in the rat and monkey*

The half-life of gastric emptying in the rat under the conditions used in these experiments was found to be 6.29 ± 1.01 min which is in close agreement with that found by Bridges, Dent & Johnson (1976). The addition of meptazinol to the marker solution used to measure emptying rate resulted in a slight but not statistically significant effect on the half-life, increasing it to 8.08 ± 1.03 (see Table 2).

The 'normal' half-life of gastric emptying in the monkey was found to be considerably longer than in the rat, being approximately 30 minutes. This agrees well with that observed in Rhesus monkeys by Jacoby & Brodie (1967). The addition of meptazinol to the marker solution resulted in the half-life of emptying being increased to 64 min (see Table 3). This difference was statistically significant ($P$ = 0.05–0.02).

## Discussion

Recently, several workers have drawn attention to the importance of gastric emptying in determining the rate

**Table 1** Plasma levels of total radioactivity and radioactivity due to unchanged meptazinol found in two female monkeys following intragastric or intraduodenal administration of $^3$H labelled drug at 25 mg/kg

| | *Monkey 1* | | | | *Monkey 2* | | | |
|---|---|---|---|---|---|---|---|---|
| *Time after dosing (h)* | *Intragastric* | | *Intraduodenal* | | *Intragastric* | | *Intraduodenal* | |
| | *Total radioact.* | *Unchanged drug* | *Total radioact.* | *Unchanged drug* | *Total radioact.* | *Unchanged drug* | *Total radioact.* | *Unchanged drug* |
| 0.25 | — | — | | | | | 54.47 | 0.472 |
| 0.50 | 0.11 | <0.010 | 45.08 | 0.297 | 0.92 | <0.006 | 53.62 | 0.470 |
| 1.00 | 0.70 | <0.010 | 37.09 | 0.204 | 0.92 | <0.006 | 38.05 | 0.315 |
| 1.50 | 1.32 | <0.010 | 28.08 | 0.145 | 0.85 | <0.006 | 22.64 | 0.151 |
| 2.00 | 5.64 | 0.014 | 20.77 | 0.107 | 1.86 | <0.006 | 17.10 | 0.148 |
| 2.50 | 11.98 | 0.025 | 13.25 | 0.073 | 3.64 | <0.006 | 13.14 | 0.096 |
| 3.00 | 15.96 | 0.034 | 10.48 | 0.057 | — | — | — | — |
| 3.50 | 28.65 | 0.067 | 8.66 | 0.048 | — | — | — | — |
| 3.75 | — | — | — | — | 9.64 | 0.009 | — | — |
| 4.00 | — | — | — | — | — | — | 5.43 | 0.036 |
| 5.00 | 24.44 | 0.037 | 4.86 | 0.032 | 4.58 | 0.008 | 3.41 | 0.019 |
| 6.00 | 15.07 | 0.026 | 3.91 | 0.028 | 4.78 | 0.007 | 2.95 | 0.014 |
| 7.00 | 9.69 | 0.019 | 3.68 | 0.026 | 4.00 | <0.006 | 2.67 | 0.011 |
| 8.00 | 7.27 | 0.018 | 3.41 | 0.027 | — | | | |

All results expressed as µg equivalents meptazinol/ml plasma.

of drug absorption and the consequential effect on the magnitude of the plasma concentration. Goto, Tsuzuki & Iguchi (1971), found increased plasma levels of aminopyrine in rabbits when the drug was given in combination with barbitone. This was due to the action of barbitone reducing the inhibitory effect of aminopyrine on gastric emptying (Goto, Tsuzuki & Iguchi, 1972). In studies with paracetamol, Heading, Nimmo, Prescott & Tothill (1973) found a good correlation between gastric emptying rate and the magnitude of the peak plasma levels of the drug in humans.

The results of the present study have implicated gastric emptying as a major determinant of the rate of absorption and magnitude of the plasma concentrations of meptazinol, since intraduodenal dosage to monkeys resulted in much more rapid absorption and higher plasma concentrations than when the drug was given intragastrically. These higher plasma concentrations were not considered to be the result of a greater chemical stability of the drug in the duodenum since the compound is known to be stable in acidic and basic environments. More probably this effect was the result of a change in the rate of absorption leading to saturation of the glucuronide conjugation mechanism. Indeed the proportion of unchanged drug to metabolites in the plasma was greater following intraduodenal dosage lending support to this hypothesis. Recently other workers have demonstrated that the glucuronide conjugation mechanism is saturable within the therapeutic dose range for a number of drugs including salicylamide and aspirin (Levy, 1971). Furthermore, Cohen, Bakke & Davies (1974) in studying the first pass metabolism of paracetamol by the isolated perfused liver of the rat showed that the hepatic extraction ratio decreased with increasing concentration of the drug in the perfusion fluid. They suggested that gastric emptying, by controlling the concentration of drug in the portal vein, would influence the amount of first pass metabolism and presumably therefore, the peak plasma levels of the compound.

The influence of gastric emptying on the extent of the first pass effect may also explain the previously observed species difference in plasma levels of meptazinol (Franklin & Aldridge, 1976). In the rat where plasma drug levels were much higher than in the monkey, gastric emptying was shown to be intrinsically more rapid and was not retarded by the action of the drug. This would be expected to lead to faster absorption, a prediction borne out experimentally since peak plasma levels were shown to

**Table 2** Influence of meptazinol on gastric emptying in female rats

| *Time after dosing (min)* | *Undosed control groups %[$^{14}$C]-PEG remaining in stomach* | *Dosed groups* %[$^{14}$C]-PEG remaining in stomach* |
|---|---|---|
| 10 | 37.85 ± 3.72 | 40.85 ± 3.60 |
| 20 | 24.39 ± 3.86 | 30.93 ± 3.62 |
| 30 | 6.90 ± 2.45 | 8.90 ± 2.11 |
| $T_{\frac{1}{2}}$ (min) | 6.29 ± 1.01 | 8.08 ± 1.03 |

Results are means ± s.e. mean for groups of 8 rats. Half-lives calculated from regression analysis of all individual (not mean) data points.

* [$^{14}$C]-polyethylene glycol ([$^{14}$C]-PEG) marker solution contained meptazinol at a dose equivalent to 25 mg/kg.

**Table 3** Influence of meptazinol on gastric emptying in female monkeys

| *Monkey No.* | *Undosed control group %[$^{14}$C]-PEG remaining in stomach after 30 min* | $T_{\frac{1}{2}}$ *(min)* | *Dosed group* %[$^{14}$C]-PEG remaining in stomach after 30 min* | $T_{\frac{1}{2}}$ *(min)* |
|---|---|---|---|---|
| 284 | 56.17 | 37.78 | 64.30 | 49.42 |
| 285 | 42.76 | 23.64 | 67.39 | 65.44 |
| 286 | 53.70 | 33.97 | 79.00 | 88.62 |
| 287 | 46.88 | 28.17 | 67.76 | 53.72 |
| | Mean ± s.e. mean = | 30.89 ± 3.12 | Mean ± s.e. mean = | 64.32 ± 8.77 |

* [$^{14}$C]-polyethylene glycol ([$^{14}$C]-PEG) marker solution contained meptazinol at a dose equivalent to 25 mg/kg.

*Note* Each value is the mean of two separate experiments.

occur by 0.66 h in the rat but not until 2.5 h in the monkey (Franklin & Aldridge, 1976). As a consequence of this more rapid absorption, saturation of the conjugation mechanism may well have occurred resulting in the observed higher plasma concentrations of the drug.

The mechanism by which meptazinol retards gastric emptying in the monkey is presently unknown. Since the drug possessed no cholinolytic activity in the rat or guinea-pig, as determined by its effects on the acetylcholine-stimulated isolated ileum (Goode, unpublished), it seems unlikely that it would do so in the monkey. However, this possibility has not been entirely excluded. It is perhaps more likely that the retarded gastric emptying was due to the local anaesthetic activity of the drug. The inhibitory action of chloroquine on gastric emptying has previously been ascribed to its local anaesthetic properties (Varga, 1966). Although meptazinol was only weakly active in this respect (equivalent to 1/10th lignocaine in the guinea-pig weal test) the relatively high concentrations of the drug in the stomach and the intrinsically slower gastric emptying in the monkey, favouring penetration of the drug to the receptor site(s), may facilitate such an action.

These observations suggest that gastric emptying may play an important role in determining the oral potency of drugs subject to extensive first pass metabolism and that drugs which retard gastric emptying are likely to exacerbate the extent of this first pass effect. Amongst the other major analgesics, morphine and pentazocine both delay gastric emptying (Crone & Ardran, 1957; Danhof, 1967), and both are subject to extensive first pass metabolism (Beckett, Taylor & Kourounakis, 1970; Brunk & Delle, 1974). It is noteworthy, in this respect that these drugs too, are much less potent after oral than after parenteral administration (Beecher, Keats, Mosteller & Lasagna, 1953; Beaver, 1968).

I am grateful to Mr Brian Morrison for his skilled technical assistance with this work and to Dr B. Alps for his cannulation of the monkey duodenum. I would also like to thank Dr A. White for the purification of the tritiated form of the drug and to express my gratitude to Drs Sanford and Swaisland for helpful discussions.

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*(Received July 20, 1976.*
*Revised November 23, 1976.)*

*Br. J. Pharmac.* (1977), 59, 571–575

# RENAL BLOOD FLOW AUTOREGULATION AND RENAL VENOUS PROSTAGLANDINS IN THE PUMP-PERFUSED CANINE KIDNEY (*In situ*)

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1 Renal autoregulation of blood flow was re-examined in the pump-perfused canine kidney and concentrations of prostaglandins E and F in the renal venous plasma were measured by radioimmunoassay.

2 At low perfusion pressures, below the range of autoregulation, prostaglandin E and F concentrations rose and calculated prostaglandin E secretion rate fell.

3 Meclofenamate (10 mg/kg, i.v.) reduced renal blood flow and prostaglandin E and F secretion rates, but did not abolish autoregulation.

4 Renal prostaglandins do not appear to mediate autoregulation in the kidney but may affect the level at which flow is controlled.

## Introduction

The mechanism of renal blood flow autoregulation has escaped elucidation despite many attempts to implicate such factors as plasma skimming (Kinter & Pappenheimer, 1956), myogenic responses (Waugh, 1958) and tissue pressure (Hinshaw, Ballus, Day & Carlson, 1959). A capillaron model has also been proposed (Murao & Rodbard, 1976) but fails to predict why there is an upper limit to autoregulation. Herbaczynska-Cedro & Vane (1973), using pump-perfused kidneys, demonstrated the abolition of autoregulation and output of prostaglandin-like substances by indomethacin, implying prostaglandin E as a mediator of autoregulation. This was subsequently challenged. Owen, Ehrhart, Weidner, Haddy & Scott (1974) failed to abolish autoregulation in the naturally perfused kidney using indomethacin. Venuto, O'Dorisio, Ferris & Stein (1975) and Anderson, Taher, Cronin, McDonald & Schrier (1975) also failed to abolish autoregulation using meclofenamate as well as indomethacin.

This discrepancy has been assigned to a methodological difference, namely the large resting resistance to flow seen in the pump-perfused kidney (Venuto *et al.*, 1975; Anderson *et al.*, 1975). We re-examined the role of prostaglandins of the E and F series in the pump-perfused canine kidney using meclofenamate to inhibit synthesis, and radioimmunoassay to estimate renal venous concentrations of both prostaglandins.

## Methods

Ten mongrel dogs of either sex, weighing between 8 and 15.5 kg were used. They were anaesthetized with pentobarbitone (30 mg/kg, i.v.) and surgical diathermy was used in subsequent operative procedures (there was no significant blood loss). Each animal was tracheotomized and systemic blood pressure was measured from the left femoral artery. Temperature was held at 37°C by a heating pad controlled by a thermistor rectal probe. A midline laparotomy was performed and a catheter placed in the left renal vein via a femoral vein, for renal venous blood sampling. The left ovarian or spermatic vein was ligated. Hypotonic saline (0.8% w/v NaCl solution) was infused intravenously at about 2 ml/min to ensure adequate urine flow. The animal was injected with heparin (1000 u/kg) and blood removed from the right carotid artery by a slowly revolving Watson Marlow MHRE pump. Part of this flow passed through a cannulating flow probe (Statham, 2.00 mm lumen) and was carried by silicone tubing through an incision in the abdominal wall to the catheterized left renal artery. Renal artery pressure (RAP) was measured at the tip of this catheter through a fine tube.

The remaining flow was shunted through a Starling resistance to a femoral vein. Using compressed air, with a controllable leak, the pressure in the Starling resistance box and so the mean perfusion pressure of the kidney could be controlled, while the pulse pressure remained about 25 mmHg. With this system

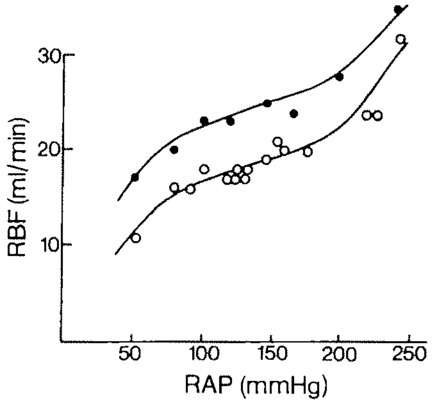

**Figure 1** This figure shows the individual results of one experiment (expt. 7) before (●) and after (O) meclofenamate. RBF = renal blood flow; RAP = renal artery pressure. The lines through the points were obtained from the cubic expression of each set of points; $y = 24.9 + 0.040(x-141) + 5.26(x-141)^3$ for control and $y = 18.5 + 0.037(x-137) + 6.46(x-137)^3$ for the meclofenamate curve.

the kidney could be perfused at a range of pressures while the pump ran at a constant slow speed, thus preventing damage to the blood at high pump speeds.

All pressures were measured with Consolidated Electrodynamic L223 transducers and the electronically averaged pressures and renal blood flow signals were recorded on light-sensitive paper (Honeywell, Visicorder 2206). Pressure flow curves were obtained by varying RAP every 5 min, since autoregulation was complete within 3 minutes. After a control curve was obtained, meclofenamate (10 mg/kg, i.v.) dissolved in 10 ml of 0.9% saline was injected and after 15 to 20 min another pressure flow curve was recorded.

Samples of renal venous blood (10 ml) were removed at 50, 100, 150 and 200 mmHg during the determination of the control pressure flow curve and at 100 mmHg after meclofenamate in 4 experiments. The samples were collected in ice cold centrifuge tubes and spun at 4°C for 25 min at 15,000 *g*. The haematocrit of each sample was noted for estimation of renal plasma flow and so prostaglandin secretion rate. The plasma was stored in a freezer for less than 3 weeks before the assay. The plasma was acidified and the prostaglandins extracted and purified by silicic acid column chromatography before radioimmunoassay.

Prostaglandin antibody was raised in rabbits with prostaglandin $E_2$ conjugated to thyroglobulin by means of the carbodiimide reaction. The antibody exhibited a cross-reaction of 100% with prostaglandin $E_1$, 2% with prostaglandin A and B groups and 5% with 13, 14 dihydro-15-keto prostaglandin $E_2$ and was used at a final concentration of 1/2,000. The sensitivity was 30 pg/tube (60 pg/ml of sample), with an intra-assay precision of about 15%.

Prostaglandin F was measured by means of the antibody raised in rabbits to prostaglandin $F_{2\alpha}$ bovine serum albumin conjugate (Dighe, Emslie, Henderson, Rutherford & Simon, 1975). The antibody showed a cross-reaction of 100% with prostaglandin $F_{1\alpha}$, 2.6% with $F_{2\beta}$ and 2% with $D_2$. The other prostaglandin groups and metabolites cross-reacted less than 1%. The antibody was used at a final dilution of 1/35,000 and the sensitivity was 45 pg/tube (90 pg/ml of sample), with an intra-assay precision of about 15%. Recovery of both prostaglandins was about 60% and the reported levels were corrected for recovery.

The pressure flow curves were plotted for each individual experiment after fitting the data by computer to a cubic expression, which describes the general shape of the autoregulation curve:

$$y = p + q(x-m) + r(x-m)^3$$

where p is the flow during autoregulation (point of inflexion), m is the pressure at this point, q is the slope of the plateau part of the autoregulation curve, r is a constant, x is the pressure at any point and y is the flow at any point.

Figure 1 demonstrates the results of one experiment (expt. 7) to which the cubic expression has been fitted by the computer using the method of least squares.

A straight regression line was also fitted to the experimental points. In the absence of autoregulation, the plateau gradient (q) would approximate to the regression coefficient ($\beta$) such that $q/\beta$ would tend to unity. Values of $q/\beta$ less than one or even negative were taken as objective evidence for autoregulation.

Values are expressed as means ± s.e. and the results were analysed by Student's $t$ test; probabilities less than 5% were accepted as being statistically significant.

## Results

Mean systolic pressure (101 ± 5 mmHg) was well maintained throughout each experiment. Two experiments showed no autoregulation and a third lost autoregulation before completion of the control period. In the remaining 7 experiments autoregulation was obtained. Mean autoregulation flow was 54 ± 13 ml/min ($n=7$) and meclofenamate (10 mg/kg, i.v.) reduced it to 26 ± 6 ml/min ($n=6$, $P<0.05$). Autoregulation was lost in one experiment after meclofenamate. The mean autoregulation flow corrected for kidney weight was 1.6 ± 0.4 ml/min per g kidney, which is about 30% lower than that reported by Ono, Kokubun & Hashimoto (1974).

Midpoint autoregulation pressure (m) did not change after meclofenamate, 133 ± 5 mmHg before and 144 ± 10 mmHg after. The ratio $q/\beta$ was always less than unity before and after meclofenamate (except in one experiment where autoregulation was lost after meclofenamate), indicating preservation of autoregulation. See Table 1 for the haemodynamic data.

There was a tendency for renal venous prostaglandin E concentrations to rise and calculated secretion rates (the product of the concentrations and their respective renal plasma flows) to fall as RAP was reduced. Using absolute values no significance could be demonstrated due to the large variation in values between experiments (Table 2). By expressing the values at 50, 100 and 150 mmHg as ratios of those at 200 mmHg, a statistically significant rise in concentration of prostaglandin E was seen at the lowest pressure of 50 mmHg ($P<0.05$) and the calculated secretion rate fell ($P<0.01$).

Prostaglandin F concentrations expressed in a similar manner also rose as pressure fell, reaching statistical significance at the lowest pressure ($P<0.05$). However, prostaglandin F secretion did not change.

Meclofenamate (10 mg/kg) produced a significant

**Table 1** Renal haemodynamics before and after meclofenamate

| *Experiment* | *m* | *q* | *β* | *p* | *q/β* |
|---|---|---|---|---|---|
| 1A | 116 | 0.012 | 0.076 | 29.2 | 0.158 |
| 1B | 132 | 0.057 | 0.095 | 13.4 | 0.600 |
| 2A | 133 | −0.027 | 0.036 | 25.4 | −0.750 |
| 2B | 184 | 0.025 | 0.033 | 10.7 | 0.757 |
| 3A | 120 | 0.016 | 0.180 | 33.9 | 0.089 |
| 3B | 155 | 0.147 | 0.175 | 25.9 | 0.840 |
| 4A | 150 | 0.070 | 0.360 | 73.0 | 0.194 |
| 4B | 113 | 0.111 | 0.229 | 41.5 | 0.484 |
| 5A | 145 | 0.059 | 0.538 | 112.7 | 0.110 |
| 5B | 144 | 0.048 | 0.130 | 46.3 | 0.369 |
| 6A | 127 | 0.060 | 0.261 | 80.8 | 0.229 |
| 6B | (42) | (0.24) | 0.17 | (6.97) | (1.43) |
| 7A | 141 | 0.040 | 0.080 | 24.9 | 0.560 |
| 7B | 137 | 0.037 | 0.083 | 18.5 | 0.446 |

A–control values; B–values after meclofenamate. m = autoregulation midpoint pressure (mmHg); q = slope of autoregulation point of curve; $\beta$ = linear regression coefficient; p is the autoregulation flow (ml/min); $q/\beta$ is an index of autoregulation. In the absence of autoregulation this value tends to unity.

In expt. 6 autoregulation was lost after meclofenamate and the pressure-flow curve becoming virtually rectilinear. The derived parameters except $\beta$ have little meaning.

**Table 2** Prostaglandin E (PGE) and prostaglandin F (PGF) renal venous plasma concentrations and calculated secretion rates at different renal artery pressures (RAP).

| *RAP (mmHg)* | n | *PGE concentration* *pg/ml* | *ratios* | *PGE secretion rate* *ng/min* | *ratios* |
|---|---|---|---|---|---|
| 50 | 6 | 542 ± 222 | 1.40 ± 0.17* | 11.31 ± 4.83 | 0.65 ± 0.12† |
| 100 | 5 | 311 ± 47 | 1.11 ± 0.25 | 10.77 ± 2.91 | 0.80 ± 0.21 |
| 150 | 5 | 398 ± 127 | 1.04 ± 0.17 | 19.47 ± 7.71 | 0.97 ± 0.25 |
| 200 | 6 | 360 ± 110 | 1 | 15.99 ± 4.63 | 1 |
| | | *PGF concentration* *pg/ml* | *ratios* | *PGF secretion rate* *ng/min* | *ratios* |
| 50 | 4 | 800 ± 353 | 2.24 ± 0.49* | 17.53 ± 7.24 | 1.04 ± 0.18 |
| 100 | 4 | 237 ± 67 | 1.07 ± 0.33 | 10.79 ± 3.84 | 0.92 ± 0.30 |
| 150 | 4 | 363 ± 105 | 1.33 ± 0.47 | 25.20 ± 9.07 | 1.82 ± 0.72 |
| 200 | 4 | 368 ± 173 | 1 | 16.37 ± 5.62 | 1 |

Absolute values are given and ratios relative to those values at 200 mmHg. *n* is the number of observations.
* $P<0.05$ † $P<0.01$

fall in the calculated secretion rate of prostaglandin E, at a perfusion pressure of 150 mmHg, from 15.65 ± 4.43 ng/min ($n = 8$) to 3.61 ± 0.61 ng/min ($n = 8$, $P < 0.05$). Renal venous plasma concentration also fell, from 315 ± 58 pg/ml to 178 ± 26 pg/ml ($P < 0.05$). Prostaglandin F showed a similar response, with a fall in the secretion rate from 18.93 ± 5.44 ng/min ($n = 7$) to 2.73 ± 0.27 ng/min ($n = 7$, $P < 0.01$) and in concentration from 314 ± 57 pg/ml to 134 ± 23 pg/ml ($P < 0.01$).

## Discussion

Renal blood flow in the anaesthetized dog is dependent on prostaglandin synthesis, particularly of prostaglandin $E_2$ (Lonigro, Itskovitz, Crowshaw & McGiff, 1973). Infusion of the prostaglandin E and A series into the canine renal artery increases renal blood flow (Fülgraff, Brandenbusch & Heintze, 1974; Tannenbaum, Splawinskii, Oates & Nies, 1975). Arachidonic acid also elevates flow and prostaglandin synthesis inhibition prevents this (Tannenbaum *et al.*, 1975).

Renal prostaglandins also appear to be involved in intrarenal blood flow distribution in the dog (Itskovitz, Stemper, Pacholczyk & McGiff, 1973; Chang, Splawinskii, Oates & Nies, 1975) and in the rabbit (Larsson & Ånggärd, 1974).

Acute renal ischaemia increases prostaglandin E-like material present in the renal vein (McGiff, Crowshaw, Terragno, Lonigro, Strand, Williamson, Lee & Ng, 1970). Herbaczynska-Cedro & Vane (1973) confirmed this and showed that indomethacin prevented this rise in concentration and also prevented autoregulation. This latter study implied that prostaglandin E was released in response to a lowered RAP and that this mediated autoregulation.

Beckman & Zehr (1975), using radioimmunoassay, showed that renal venous prostaglandin E concentration rose when renal blood flow was reduced, but this was due to a dilution effect. A 50% reduction in flow resulted in a rise in concentration but less than double the control concentration, which must be interpreted as a fall in secretion rate.

In calculating secretion rate, it is assumed that the arterial prostaglandins presented to the kidney are removed in passage (Aiken & Vane, 1973) so that all the renal venous prostaglandins can be assumed to have been newly synthesized by the kidney. It is possible that the intrarenal metabolism of prostaglandins is flow-dependent, decreasing in efficiency as the flow increases.

In the naturally perfused kidney, prostaglandin synthetase inhibitors do not abolish renal blood flow autoregulation (Owen *et al.*, 1974; Venuto *et al.*, 1975; Anderson *et al.*, 1975). The latter two groups suggest that the large resting resistance seen in the pump-perfused kidney (Herbaczynska-Cedro & Vane, 1973) may lead to prostaglandin-dependent autoregulation. This cannot be the case since despite inhibition of prostaglandin synthesis, 6 out of 7 of our experiments still showed autoregulation. Prostaglandin E secretion was reduced by about 70%, in agreement with Venuto *et al.* (1975). It is possible that although secretion rate was greatly reduced, the smaller reduction in concentration (due to the concomitant fall in flow) was not enough to abolish autoregulation. It is possible that intrarenal blood prostaglandin concentration rather than secretion rate *per se* is important in determining intrarenal haemodynamics.

In the conscious dog, indomethacin (2 mg/kg) did not affect blood pressure, renal blood flow or intrarenal blood flow distribution (Zins, 1975) and meclofenamate and the competitive synthetase inhibitor Ro 20–5720 (1 mg/kg) did not alter blood pressure or renal blood flow (Kirschenbaum & Stein, 1976). However, in these studies, inhibition was not examined.

Renal blood flow and intrarenal haemodynamics may be dependent on prostaglandin synthesis in the anaesthetized dog due to the elevated prostaglandin E concentrations. In 7 conscious dogs we have obtained resting renal venous plasma concentrations of prostaglandin E of 191 ± 25 pg/ml ($n = 10$), levels significantly lower than those reported in the anaesthetized dogs at a pressure of 100 mmHg, 361 ± 63 pg/ml ($n = 10$, $P < 0.05$). Levels may be higher in the anaesthetized animal for several reasons. The high resting resistance in the pump-perfused kidney will lower flow and so raise concentrations through a diluting mechanism. Angiotensin is released during barbiturate anaesthesia and this lowers renal blood flow (Burger, Hopkins, Tulloch & Hollenburg, 1976) and will also cause a direct release of prostaglandins (McGiff, Crowshaw, Terragno & Lonigro, 1970).

The mere introduction of the superfusion bioassay system as used by Herbaczynska-Cedro & Vane (1973) has been reported to elevate arterial and renal venous prostaglandin E, possibly due to kinin formation (Satch & Zimmerman, 1976). It is possible that foreign surfaces in general, such as those of the pump circuit tubing, may have a similar effect.

In conclusion, renal prostaglandins do not appear to mediate autoregulation of renal blood flow in the pump-perfused kidney, but may play a role in determining absolute blood flow, at least in the anaesthetized dog.

We would like to thank Mr P.H. Whelpdale for his excellent technical assistance and Dr R. Elton for his statistical advice and help. G.W.S. is an MRC scholar.

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*(Received July 26, 1976.*
*Revised November 8, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 577–583

# PROFILE OF $\beta$-ADRENOCEPTORS IN FEMORAL, SUPERIOR MESENTERIC AND RENAL VASCULAR BEDS OF DOGS

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1 The homogeneity of $\beta$-adrenoceptors in femoral, superior mesenteric and renal vascular beds was investigated by the use of the regional perfusion technique in dogs.

2 Isoprenaline and salbutamol produced dose-related increases in femoral and superior mesenteric blood flow. The dose-response curves for the two agonists were parallel, but salbutamol was approximately 1/15 as potent as isoprenaline on a weight basis.

3 Isoprenaline and salbutamol increased renal blood flow in a dose-related manner. However, salbutamol was approximately 1/240 as potent as isoprenaline on a weight basis, and the slope of the dose-response curve for salbutamol was less steep than that for isoprenaline.

4 The dose-response curves to isoprenaline for increase in femoral and superior mesenteric blood flow were shifted to the right by intravenous pindolol but not by intravenous or intra-arterial practolol.

5 The dose-response curves to isoprenaline for increase in renal bood flow were shifted to the right more markedly by intravenous pindolol than by intravenous or intra-arterial practolol.

6 The results indicate that $\beta$-adrenoceptors of the renal vascular bed consist of $\beta_1$-type and $\beta_2$-type whereas the femoral and superior mesenteric vascular beds contain only $\beta_2$-adrenoceptors.

## Introduction

Lands and his co-workers (Lands, Arnold, McAuliff, Luduena & Brown, 1967a; Lands, Luduena & Buzzo, 1967b) proposed that $\beta$-adrenoceptors could be divided into a $\beta_1$-group in cardiac muscle and a $\beta_2$-group in bronchial and vascular smooth muscle. Recent work suggests that not only do $\beta$-adrenoceptors in bronchial and vascular smooth muscle differ from those in cardiac muscle but that $\beta$-adrenoceptors in the former two sites are also not homogeneous (Bristow, Sherrod & Green, 1970; Wasserman & Levy, 1974; Wardell, Colella, Shetzline & Fowler, 1974). Little is known about the homogeneity of $\beta$-adrenoceptors in the vasculature. The present experiments were designed to test this point. For this purpose the femoral, superior mesenteric and renal vascular beds were perfused by the use of the regional perfusion technique (Hashimoto & Kumakura, 1965) in dogs anaesthetized with pentobarbitone.

## Methods

Adult mongrel dogs of either sex, weighing 15–18 kg, were anaesthetized with pentobarbitone sodium initially at a dose of 30 mg/kg (i.v.) and received heparin sodium at a dose of 500 units/kg (i.v.). Subsequently pentobarbitone sodium was infused intravenously at a rate of 4 mg kg$^{-1}$ h$^{-1}$ and heparin sodium at a rate of 100 units kg$^{-1}$ h$^{-1}$ by the use of an infusion pump (Harvard Apparatus, Model 600). The number of dogs used was as follows: 26 for experiments on renal circulation, 15 for those on superior mesenteric circulation and 15 for those on femoral circulation. The surgical procedures were essentially the same as those described by Hashimoto & Kumakura (1965). The left renal, superior mesenteric or right femoral artery was cannulated and blood from the right femoral artery was conducted to the cannulated artery by means of a peristaltic pump (Harvard Apparatus, Model 1210). Constant pressure perfusion was achieved by the use of Starling

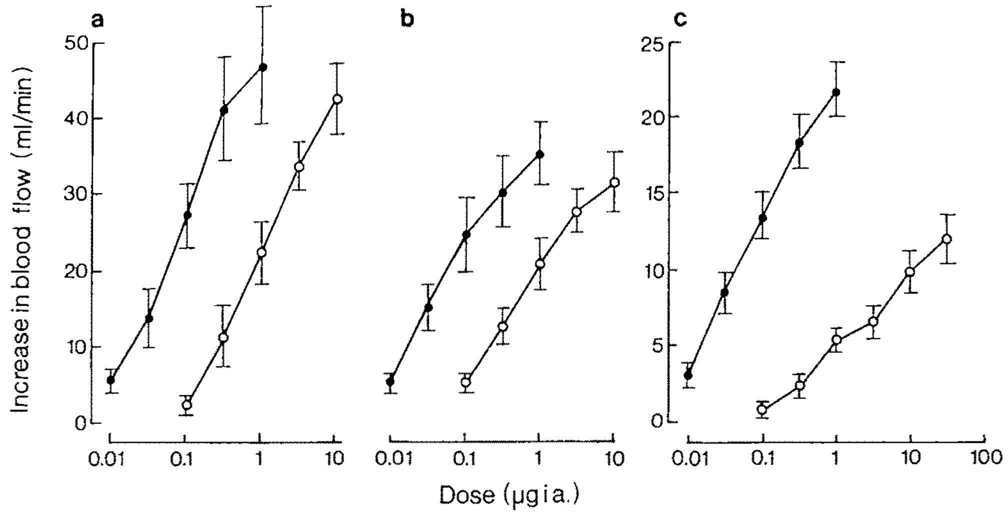

**Figure 1** Dose-response curves to intra-arterial isoprenaline (●) and salbutamol (○) for peak increase in (a) femoral, (b) superior mesenteric and (c) renal blood flow in dogs. Each symbol represents the mean and vertical bars show s.e. mean. Number of experiments is 11 for isoprenaline in renal circulation and 5 in the remainder.

pneumatic resistance through which a portion of blood was shunted to the left jugular vein. Perfusion pressure was maintained at 120 mmHg for renal circulation and at 100 mmHg for superior mesenteric circulation and for femoral circulation. Blood flow in each case was measured by an electromagnetic flowmeter (Nihon Koden, MF-26). The systemic blood pressure was measured from the left carotid artery by a pressure transducer (San-ei Instrument, MPU-0.5) and the heart rate by a cardiotachometer (San-ei Instrument, Type 2130) triggered by blood pressure pulses or R waves of the ECG. All the recordings were made on an ink-writing rectigraph (San-ei Instrument, Rectihoriz Type 8S).

The drugs used in this study were (–)-isoprenaline hydrochloride (Nikken Kagaku), (±)-salbutamol sulphate (Leiras), (±)-noradrenaline hydrochloride (Sankyo), (±)-pindolol (Sandoz), (±)-practolol (ICI) and phenoxybenzamine hydrochloride (Nakarai Kagaku). All drugs except for pindolol and practolol were dissolved in 0.9% w/v NaCl solution (saline). Pindolol was dissolved in equimolar maleic acid solution and practolol in equimolar HCl. All drug solutions were diluted with saline to the desired concentrations Doses of all drugs except for pindolol and practolol refer to their salts, and those of the latter two drugs to their bases. Agonist solutions in a volume of 10–30 µl were injected into the rubber tubing connected to the arterial cannula within 3 s by the use of microsyringes. Antagonist solutions were infused at a rate of 0.2 ml/min into the rubber tubing connected to the arterial cannula by the aid of an infusion pump (Harvard Apparatus, Model 600) or injected into the forearm vein (0.4–1.0 ml in 10 s).

Values in the text are arithmetic means ± s.e. The difference between mean values was analysed by Student's *t* test and judged to be significant when *P* values $< 0.05$.

## Results

### *Effects of isoprenaline and salbutamol on femoral, superior mesenteric and renal blood flow*

Femoral and superior mesenteric blood flow at a perfusion pressure of 100 mmHg was 46 ± 7 ml/min ($n = 15$) and 108 ± 7 ml/min ($n = 15$), respectively. Intra-arterial injections of isoprenaline (0.01–1 µg) and of salbutamol (0.1–10 µg) increased blood flow through both arteries in a dose-related manner. Dose-response curves to isoprenaline and to salbutamol for peak increases in blood flow were parallel in both circulations (Figure 1a,b). When compared on the basis of doses (ED, 20 ml/min) increasing blood flow by 20 ml/min, the potency of salbutamol was almost 1/15 that of isoprenaline on a weight basis in the two vascular beds.

In all experiments on renal circulation the animals were initially given phenoxybenzamine (2–5 mg/kg i.v.). Under these conditions renal blood flow at a perfusion pressure of 120 mmHg was 106 ± 4 ml/min ($n = 26$) and decreases in renal blood flow in response to noradrenaline (0.1–0.3 µg i.a.) were greatly diminished. Intra-arterial injections of isoprenaline (0.01–1 µg) and of salbutamol (0.3–30 µg) produced a dose-related increase in the

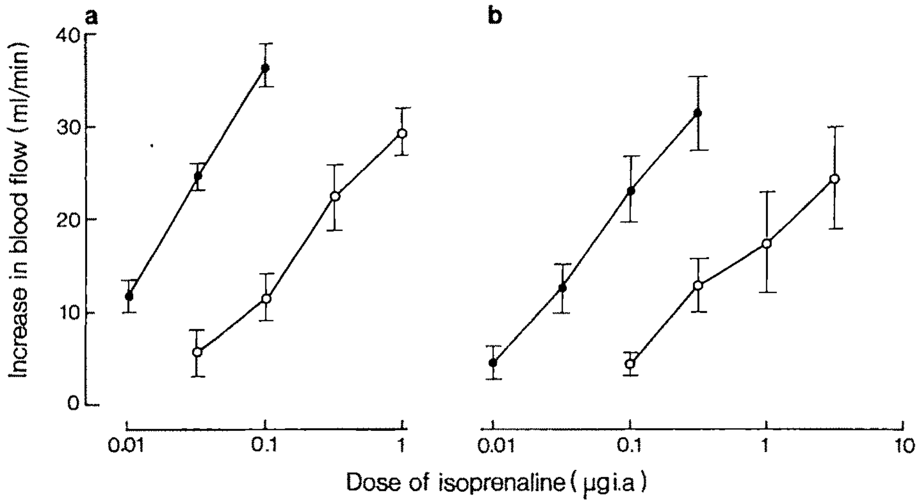

**Figure 2** Dose-response curves to isoprenaline for increase in (a) femoral blood flow and (b) superior mesenteric blood flow before (●) and after (○) a single injection of pindolol (3 μg/kg i.v.). Vertical bars show s.e. mean ($n = 5$).

renal blood flow. Figure 1c shows dose-response curves to the two agonists for peak increase in blood flow. As seen in this figure, the slope of the dose-response curve for salbutamol was less steep than that for isoprenaline. When compared on the basis of doses (ED, 10 ml/min) increasing the renal blood flow by 10 ml/min, the potency of salbutamol was approximately 1/240 that of isoprenaline on a weight basis. These doses, apart from the highest doses used of isoprenaline and salbutamol injected intra-arterially, had almost no systemic effect.

### *Differential modification by pindolol and practolol of the effect of isoprenaline on femoral and superior mesenteric blood flow*

The dose-response curves to isoprenaline for increases in femoral and superior mesenteric blood flow were shifted almost in a parallel fashion to the right by intravenous injections of pindolol (3 μg/kg) (Figure 2a, b). In contrast, the dose-response curves to isoprenaline for increase in femoral blood flow were not altered significantly by intravenous injections of practolol (0.3 and 3 mg/kg) (Figure 3a). The dose-response curves to isoprenaline for increase in superior mesenteric blood flow were also not modified by intra-arterial infusions of practolol (100 and 300 μg/min) (Figure 3b).

### *Differential modification by practolol and pindolol of the effects of isoprenaline, and salbutamol on renal blood flow*

Intravenous injections of practolol (0.3 and 3 mg/kg), which antagonized the positive chronotropic effect of 0.1 μg/kg of intravenous isoprenaline by 60 to 100% but did not significantly alter the vasodepressor effect, shifted the dose-response curve to isoprenaline (0.01–3 μg i.a.) for increase in renal blood flow in an almost parallel fashion to the right (Figure 4a) but did not alter that to salbutamol (0.1–10 μg i.a.) (Figure 4b). Log ratios of ED, 10 ml/min for isoprenaline after 0.3 and 3 mg/kg of intravenous practolol to ED, 10 ml/min before practolol were 0.3 and 0.7

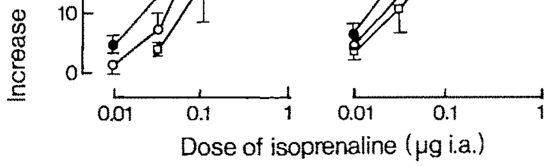

**Figure 3** (a) Dose-response curves to isoprenaline for increase in femoral blood flow before (●) and after single injections of practolol: (○) 0.3 mg/kg i.v.; (□) 3 mg/kg i.v. (b) Similar curves for increase in superior mesenteric blood flow before (●) and during infusion of practolol into the superior mesenteric artery: (○) 100 μg/min; (□) 300 μg/min. Vertical bars show s.e. mean ($n = 5$).

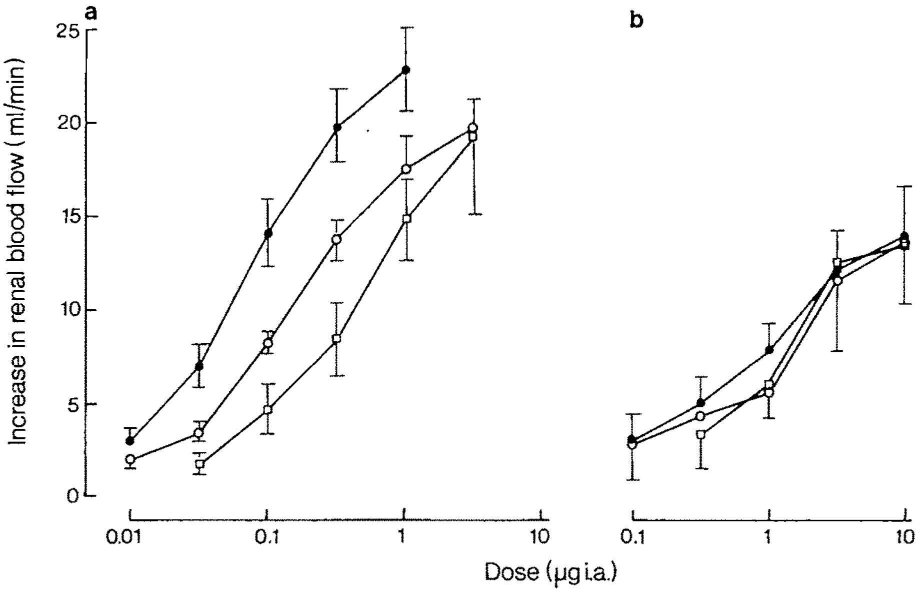

**Figure 4** Dose-response curves to (a) isoprenaline and (b) salbutamol for increase in renal blood flow before (●) and after practolol: (○) 0.3 mg/kg i.v.; (□) 3 mg/kg i.v. Vertical bars show s.e. mean ($n=5$).

respectively (Table 1), indicating that the doses of practolol produced shifts of 0.3 and 0.7 log units of the dose-response curves for isoprenaline to the right. Essentially similar antagonism of renal blood flow responses to isoprenaline (0.03–0.3 µg i.a.) was obtained with intra-arterial infusions of practolol (10–300 µg/min) (Table 1). Figure 5 illustrates typical experiments. The blocking effect of practolol was reversible; the blood flow response to isoprenaline recovered to a control level 5–20 min after interruption of infusion of practolol. Dose-response curves for isoprenaline before and during intra-arterial infusions of practolol are shown in Figure 6. In this figure, it should be noted that the response to isoprenaline at a dose as small as 0.03 µg was not completely inhibited by practolol.

**Table 1** Doses (ED, 10 ml/min) of isoprenaline increasing renal blood flow by 10 ml/min in the absence and in the presence of practolol or pindolol and log ED, 10 ml/min ratios in dogs

| *β-Adrenoceptor antagonist* | *Dose* | *ED, 10 ml/min (µg) (95% confidence limits)* | *Log ED, 10 ml/min ratio*† |
|---|---|---|---|
| Intravenous practolol | 0 | 0.05 (0.02–0.07) | 0 |
| | 0.3 mg/kg | 0.11 (0.07–0.17)* | 0.3 |
| | 3 mg/kg | 0.24 (0.14–0.44)* | 0.7 |
| Intra-arterial practolol | 0 | 0.08 (0.06–0.11) | 0 |
| | 10 µg/min | 0.15 (0.12–0.20)* | 0.3 |
| | 100 µg/min | 0.38 (0.29–0.53)* | 0.7 |
| | 300 µg/min | 0.85 (0.45–2.77)* | 1.0 |
| Intravenous pindolol | 0 | 0.07 (0.04–0.08) | 0 |
| | 3 µg/kg | 0.59 (0.38–0.95)* | 0.9 |
| | 10 µg/kg | 3.94 (2.23–10.08)* | 1.7 |

† Log ED, 10 ml/min ratio was calculated from the following equation: log ED, 10 ml/min ratio = log ED, 10 ml/min for isoprenaline in the presence of a β-adrenoceptor antagonist minus log ED, 10 ml/min for isoprenaline in the absence of a β-adrenoceptor antagonist.

* Significantly different from control values ($P < 0.05$).

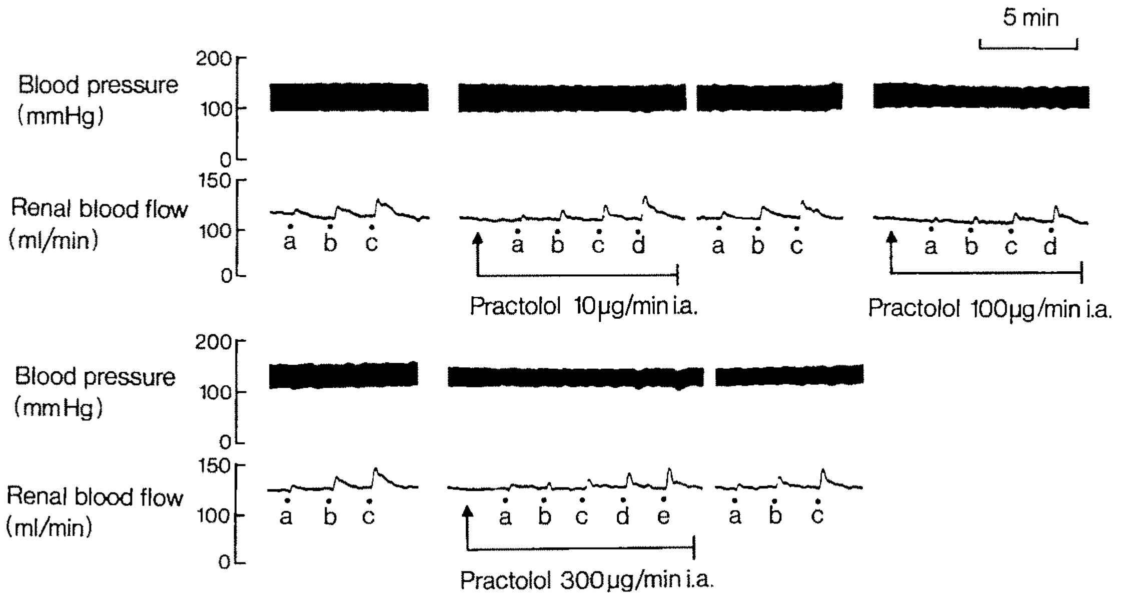

**Figure 5** Effects of isoprenaline injected into the renal artery before and during infusion of practolol into the same artery. Doses of isoprenaline injected into the renal artery were as follows: (a) 0.03 μg; (b) 0.1 μg; (c) 0.3 μg; (d) 1 μg; (e) 3 μg.

On the other hand, intravenous injections of pindolol (3 and 10 μg/kg) shifted the dose-response curves for isoprenaline (0.03–10 μg i.a.) and for salbutamol (0.1–100 μg i.a.) to the right (Figure 7a, b). Log ratios of ED, 10 ml/min for isoprenaline after 3 and 10 μg/kg i.v. of pindolol to ED, 10 ml/min in control were 0.9 and 1.7, respectively (Table 1). These values were greater than the log ED, 10 ml/min ratios obtained with 0.3 and 3 mg/kg of intravenous practolol.

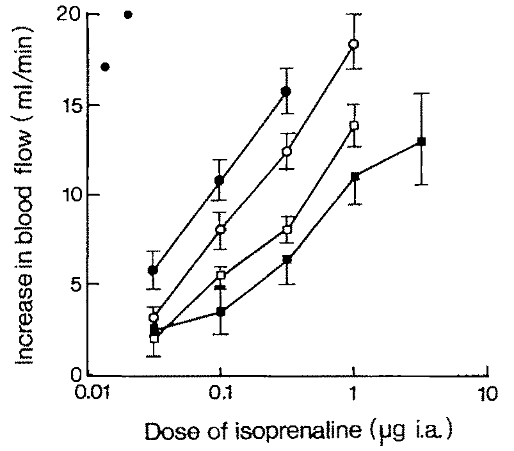

**Figure 6** Dose-response curves to isoprenaline for increase in renal blood flow before (●) and during infusion of practolol into the renal artery: (○) 10 μg/min; (□) 100 μg/min; (■) 300 μg/min. Vertical bars show s.e. mean ($n=5$).

## Discussion

In the present experiments the dose-response curves to isoprenaline and salbutamol for increase in femoral or superior mesenteric blood flow were almost parallel. In both vascular beds salbutamol was approximately 1/15 as potent as isoprenaline on a weight basis. The present results were thus consistent with those obtained by Cullum, Farmer, Jack & Levy (1969) and by Wasserman & Levy (1974) who found that salbutamol was 1/10 to 1/20 as potent as isoprenaline in increasing blood flow through or in decreasing perfusion pressure of the dog hind limb. Salbutamol is more selective on bronchial and vascular β-adrenoceptors than on cardiac β-adrenoceptors (Cullum *et al.*, 1969; Daly, Farmer & Levy, 1971; Wasserman & Levy, 1974) and belongs to selective stimulants of $\beta_2$-adrenoceptors classified by Lands *et al.* (1967a, b). In the present experiments the position of the dose-response curve to isoprenaline for increase in femoral blood flow was not significantly altered by intravenous injection of practolol and the dose-response curve to isoprenaline for increase in superior mesenteric blood flow was also not significantly

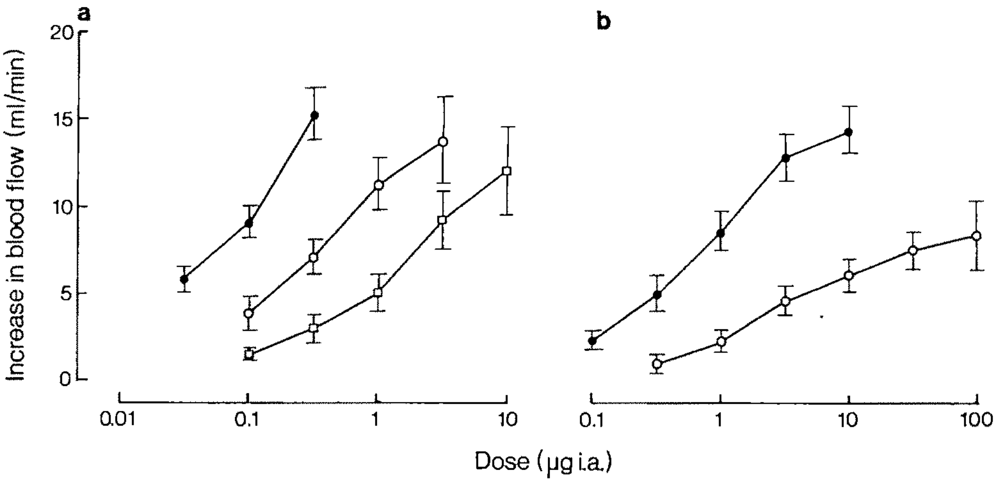

**Figure 7** Dose-response curves to (a) isoprenaline and (b) salbutamol for increase in renal blood flow before (●) and after pindolol: (○) 3 μg/kg i.v.; (□) 10 μg/kg i.v. Vertical bars show s.e. mean ($n=5$).

modified by intra-arterial infusion of practolol. In contrast, the two dose-curves for isoprenaline were effectively shifted to the right by pindolol. Practolol antagonizes the positive chronotropic and inotropic effects but not the vasodilator action of isoprenaline (Dunlop & Shanks, 1968), and can be classified as a selective $\beta_1$-adrenoceptor antagonist. Unlike practolol, pindolol blocks non-selectively $\beta$-adrenoceptors in cardiac muscle and in bronchial and vascular smooth muscle (Giudicelli, Schmitt & Boissier, 1969; Yabuuchi & Kinoshita, 1974). Thus, the present results can be interpreted in such a way that $\beta$-adrenoceptors in the femoral and superior mesenteric vascular beds are homogeneous and belong to the $\beta_2$-type proposed by Lands *et al.* (1967a,b).

In the present experiments in which the animals were treated initially with phenoxybenzamine, intra-arterial injection of isoprenaline produced an increase in renal blood flow. The results are in accord with those of McNay & Goldberg (1966). Salbutamol also increased the renal blood flow. However, the potency of salbutamol in increasing renal blood flow was approximately 1/240 that of isoprenaline on a weight basis. Thus, the difference in potency between isoprenaline and salbutamol as vasodilators in the renal vascular bed was much greater than that between the two compounds in the femoral or superior mesenteric vascular beds. Furthermore, the dose-response curve to salbutamol for increase in renal blood flow was less steep than that to isoprenaline. These observations suggest that $\beta$-adrenoceptors in the renal vascular bed may be different from those in the femoral and superior mesenteric vascular beds. The dose-response curve to isoprenaline for increase in renal blood flow was shifted almost in a parallel fashion to the right by intravenous or intra-arterial practolol in doses which did not significantly change the dose-response curves to isoprenaline for increases in femoral and superior mesenteric blood flow. However, the renal blood flow response to isoprenaline at a dose as small as 0.03 μg was not completely abolished even by intra-arterial infusion at a maximal rate of 300 μg/min of practolol used in the present experiments. A 10-fold increase in intravenous or intra-arterial doses of practolol produced a shift of approximately 0.4 log units to the right in the dose-response curves to isoprenaline for increase in renal blood flow, whereas only a 3-fold increase in intravenous doses of pindolol induced a shift of 0.8 log units. This difference in displacement of the dose-response curves for isoprenaline by pindolol and practolol may be explained on the basis of the difference in $\beta$-adrenoceptor blocking properties of pindolol and practolol, mentioned in the preceding paragraph. Furthermore the increase in renal blood flow induced by salbutamol was not antagonized by practolol but was by pindolol. The failure of practolol to modify the effect of salbutamol may be attributed to the weaker stimulant property of salbutamol on $\beta_1$-adrenoceptors than on $\beta_2$-adrenoceptors. From these results, it can be concluded that $\beta$-adrenoceptors of both the $\beta_1$-type and $\beta_2$-type proposed by Lands *et al.* (1967a,b) may be demonstrated in the renal vasculature.

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*(Received July 28, 1976.)*

*Br. J. Pharmac.* (1977), 59, 585–590

# THE FATE OF BIOGENIC MONOAMINES IN PERFUSED RABBIT LUNG

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1 Inactivation of β-phenylethylamine and several of its derivatives was studied in a preparation of rabbit lung perfused with Krebs physiological medium at 37°C. Inactivation or removal of these compounds was calculated as the difference between the concentration of each amine in the perfusion medium and the effluent, collected separately from each lung. The extent of amine metabolic degradation was also measured, by column chromatography, in lung effluent.

2 With this technique the magnitude of amine removal as a function of concentration was determined and an apparent $K_m$ and $V_{max}$ of removal were calculated for each amine.

3 Percentage removal was highest with phenylethylamine (95%), and decreased, apparently in relation to increasing phenyl- and side chain-hydroxylation (and therefore likely increased hydrophilicity), with 5-hydroxytryptamine (64%), tyramine (53%), octopamine (35%), dopamine (32%) and noradrenaline (23%).

4 Inactivation of each amine could be accounted for by metabolic degradation to deaminated products, which appeared in lung effluent within 90 s of beginning amine perfusion.

5 When intrapulmonary metabolism of phenylethylamine was inhibited by simultaneous perfusion with semicarbazide (10 mM) and pargyline (10 μM), the removal rate was unaltered, establishing that uptake of the amine from the vascular space is not dependent on metabolism at least for 4 min infusions.

## Introduction

After they leave the vascular space of perfused rabbit lung, 5-hydroxytryptamine (5-HT), noradrenaline (NA) and phenylethylamine are degraded by monoamine oxidase (MAO) and, in the case of NA, also by catechol-*O*-methyltransferase (COMT) (Hughes, Gillis & Bloom, 1969; Gillis & Iwasawa, 1972; Roth & Gillis, 1975). Three forms of MAO have been distinguished in the perfused lung. These are the A and B forms of the mitochondrial oxidase and the 'plasma' amine oxidase (Roth & Gillis, 1975). During a single passage of [$^{14}$C]-phenylethylamine through rabbit lung almost 90% of the radioactivity appearing in the effluent was associated with the product of deamination, phenylethylacetic acid; little retention of phenylethylamine or metabolite was evident in these lungs (Roth & Gillis, 1975).

There is at present little information available concerning those structural features which are important for removal (i.e., transport and/or metabolism) of phenylethylamine derivatives by the lung. Accordingly, we carried out, and describe in this paper, results of experiments in which we compared the rates of removal and extent of metabolic degradation, in the perfused lung, of phenylethylamine, tyramine, octopamine, dopamine, NA and 5-HT.

## Methods

### *Lung perfusion and chromatography*

Experiments were carried out on the rabbit lung perfused *in vitro* as described by Gillis & Iwasawa (1972). This preparation, in effect, creates paired organs for experimental purposes. In brief, left and right lungs were perfused independently at 10 ml/min with physiological medium (pH 7.4; 37°C) for a 15-min period to clear the lungs of blood. The perfusion medium had the following composition (mM): NaCl 118.07, KCl 4.75, $CaCl_2$ 2.54, $KH_2PO_4$ 0.93, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, glucose 11.1 and disodium calcium edetate 0.027. Both lungs were then perfused

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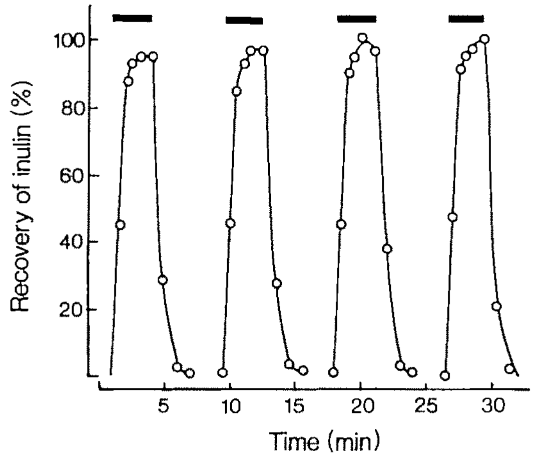

**Figure 1** Recovery of [$^{14}$C]-inulin in effluent of perfused rabbit lung. Lung was perfused at a rate of 10 ml/min for 4 consecutive periods of 4 min with $4 \times 10^5$ d/min of [$^{14}$C]-inulin/ml of Krebs medium. Shown on the ordinate scale is the inulin measured in the effluent (per 30 s) expressed as a percentage of that perfused (per 30 seconds). Horizontal bars show duration of inulin perfusion.

for 10 min with this medium to which one of the amines under study ($^{14}$C or tritium labelled) had been added. During amine perfusion, the lung effluent was collected for 1 min periods. Total radioactivity in each effluent fraction and in the perfusion medium was measured. Aliquots of lung effluent (0.5 ml) were passed through columns of Bio-Rex 70 (Bio Rad Labs, Richmond, Calif.) cation exchange resin after which the deaminated product was eluted from the columns with 2.5 ml of water (Roth & Gillis, 1975). When effluents containing NA and dopamine were analyzed, columns were subsequently eluted with 3 ml of 2% borate to remove unchanged catecholamine and then with 3 ml of 0.2 M HCl solution to remove *O*-methylated products. The radioactivity in the water, borate and acid washes from the columns was determined by liquid scintillation spectrometry. Removal of amine (per fraction) was calculated as the difference between the radioactivity present as unchanged amine both in the perfusion medium and in each fraction. Total removal is then the cumulative removal during any chosen time period of amine perfusion.

### *Estimation of initial rates of amine removal*

In some experiments we wished to determine the 'initial rates' of amine removal, in order to calculate kinetics for the overall process. Since it was not clear which fractions best reflected the initial rates of removal, it was necessary, in preliminary experiments, to determine the rate of equilibration of an extracellular marker, [$^{14}$C]-inulin, during 4 successive perfusion periods, each of 4 min duration. Results of a typical experiment (Figure 1) show that the third fraction after beginning inulin perfusion reflects 84–90% recovery of inulin in the effluent (i.e., almost complete equilibration of the inulin space) during each of the four perfusion periods. These data suggested that initial removal of amine should be calculated as [amine infused in 30 s – amine recovered in fraction 3]. In order to increase accuracy of calculated initial removal rate however, we determined the average removal for fractions 3, 4 and 5. Figure 1 also indicates that all inulin within the perfusable space of lung is removed during 3 min of perfusion with inulin-free Krebs solution.

To measure the initial rates of amine removal, lungs were perfused for 4 min with Krebs medium containing one of the $^{14}$C- or $^{3}$H-labelled amines. Amine perfusion was repeated 3 times with increasing concentrations of the same substrate. Between each period of amine perfusion, lungs were washed for 5 min with amine-free Krebs medium to clear the vascular space of accumulated radioactively-labelled material. Lung effluent fractions were collected for 30 s periods and the third, fourth and fifth fractions (collected as indicated above) after beginning each amine perfusion were used to determine amine removal.

Data derived from these studies were used to calculate the apparent $K_m$ and $V_{max}$ values for removal in left and right perfused lungs of each rabbit. This method eliminates much of the variability in removal among different lung preparations, since removal of each amine, at several different concentrations, is measured in the same lung. Apparent $K_m$ and $V_{max}$ values were determined on a Wang computer by means of a programme supplied by Wang laboratories, Tewkesbury, Mass.

### *Drugs and isotopes*

$\beta$-Phenylethylamine hydrochloride-[ethyl-1-$^{14}$C] (9.86 mCi/mmol), dopamine hydrobromide-[1-$^{14}$C] (13.69 mCi/mmol), tyramine hydrochloride-[1-$^{14}$C] (9.2 mCi/mmol), DL-octopamine-[2-$^{3}$H](N) (3.7 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. (±)-Noradrenaline (±)-bitartrate[carbinol-$^{14}$C] (35 mCi/mmol) and 5-hydroxytryptamine-[2-$^{14}$C]creatinine sulphate (59 mCi/mmol) were obtained from Amersham/Searle Corp., Arlington Heights, Ill. The unlabelled amine salts were purchased from Sigma Chemical Co., St. Louis, Mo.

## Results

The ability of rabbit lung to deaminate phenylethylamine, 5-HT, tyramine, octopamine,

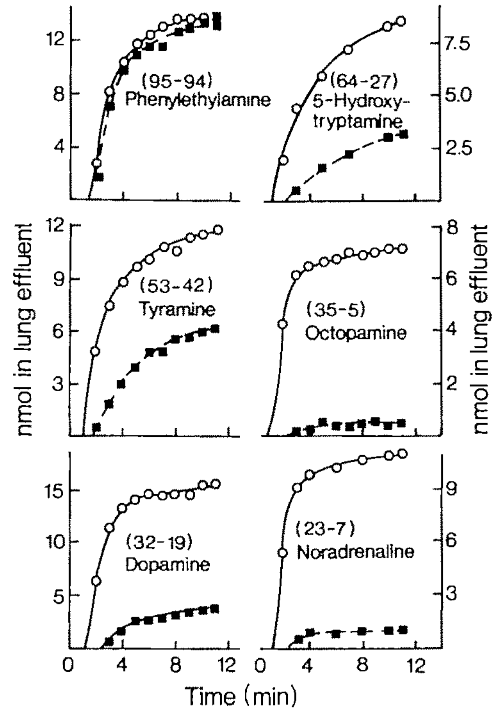

**Figure 2** Appearance of amines and metabolites in effluent from lungs perfused with different amines for 10 minutes. Data for amine *and* metabolite (O) and deaminated metabolite alone (■) are presented. For each amine, the percentage total removal and percentage total metabolism during 10 min of perfusion are given in parentheses. The concentrations (μM) of amines used were: phenylethylamine 1.6; tyramine 1.2; dopamine 1.8; 5-hydroxytryptamine 1.4; octopamine 1.0 and noradrenaline 1.5.

dopamine and NA is illustrated in Figure 2. Also indicated in parentheses in the figure, are the (percentage) total removal and total metabolism for each amine. Data presented in Figure 2 are representative of at least 4 similar experiments with each amine. In these experiments rabbit lungs were perfused with 1–2 μM of one of the radioactively-labelled amines for 10 minutes. The percentage removal of phenylethylamine was 95%, the highest observed. This is followed, in decreasing order, by removal of 5-HT (64%), tyramine (53%), octopamine (35%), dopamine (32%) and NA (23%). By comparing the figure for percentage total deaminated product appearing in the effluent with the corresponding value for percentage removal, it can be seen that, among the amines tested, phenylethylamine, tyramine and dopamine were those most effectively deaminated by perfused rabbit lung. In the cases of NA and dopamine, the percentage of radioactivity in the acid effluent of columns (COMT metabolites) was always less than 8% of the total.

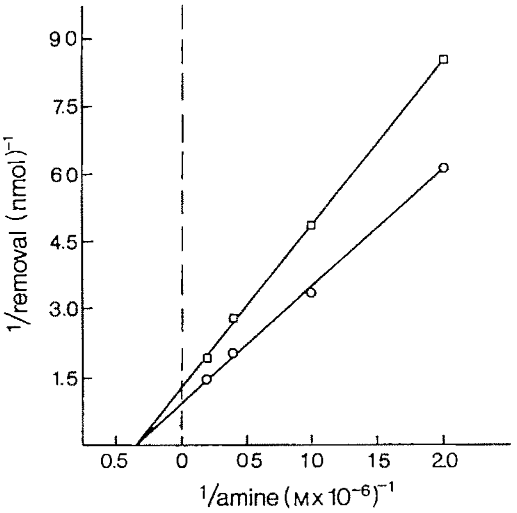

**Figure 3** Double reciprocal plot of noradrenaline (□) and 5-hydroxytryptamine (O) removal *vs* concentration.

In experiments designed to estimate initial rates of amine removal, lungs were perfused with different amine concentrations during 4 successive perfusion periods as described in the Methods section. However, it was first necessary to demonstrate that at a given concentration of amine, removal remained constant during each period of perfusion. Table 1 presents the results of two experiments in which lungs were

**Table 1** Average removal (per 30 s) during each of four successive periods of [$^{14}$C]-5-hydroxytryptamine perfusion

| *Period of amine perfusion* | *Amine removal* (nmol/30 s)* Left lung | *Right lung* |
|---|---|---|
| *Experiment 1* | | |
| 1 | 2.431 | 2.453 |
| 2 | 2.315 | 2.326 |
| 3 | 2.426 | 2.461 |
| 4 | 2.442 | 2.500 |
| *Experiment 2* | | |
| 1 | 4.509 | 4.593 |
| 2 | 4.335 | 4.433 |
| 3 | 4.284 | 4.440 |
| 4 | 4.274 | 4.510 |

5-Hydroxytryptamine concentrations were, experiment 1, 0.56 μM; experiment 2, 1.0 μM.

* Calculated as, $\sum$ (nmol$_{medium}$ − nmol$_{effluent}$) of fractions 3+4+5.

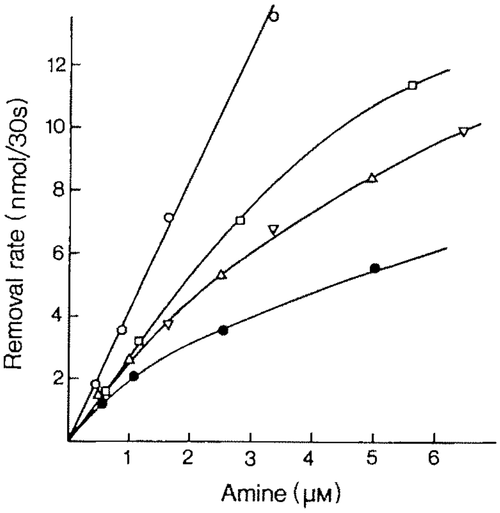

**Figure 4** The rates of amine removal as functions of the concentration perfused. Each point represents the mean of data from four lungs. (O) Phenylethylamine; (□) tyramine; (▽) octopamine; (△) dopamine; (●) noradrenaline.

perfused with either 0.56 μM or 1.0 μM [$^{14}$C]-5-HT during four successive periods as described in the Methods section. It is apparent that the magnitude of removal remained constant during each period of amine perfusion; accordingly, we felt justified in using successive periods of amine perfusion to determine 'initial' removal rates before calculating $K_m$ values for amine transport. Results of a typical experiment in which rabbit lungs were perfused with four different concentrations of 5-HT or NA are illustrated in the Lineweaver-Burk plots in Figure 3. The average $K_m$ (± s.d.) determined for 5-HT and NA was 2.7 μM 1.7 ($n = 6$) and 3.2 μM 1.1 ($n = 5$), respectively.

Pulmonary removal of the phenylethylamine derivatives, as a function of increasing concentrations perfused is illustrated in Figure 4. It is clear from this figure, and also from the data presented in Figure 2, that over the concentration range used, phenylethylamine was the most extensively removed amine and was followed in decreasing order by tyramine, dopamine, octopamine and NA. Attempts were made to analyze the data of Figure 4 according to the method of Lineweaver & Burk (1934). The 'apparent' $K_m$ and $V_{max}$ values for NA, tyramine, dopamine and octopamine are shown in Table 2. Though linear double reciprocal plots were obtained for each of these amines, the calculated $K_m$ values for the latter three amines were greater than the highest concentration perfused through the lung to estimate those $K_m$ values. However, when in additional experiments, the concentration range of each amine was raised to include that represented by the $K_m$ value shown in Table 2, the $K_m$ then calculated also fell outside this new concentration range used.

**Table 2** Apparent $K_m$ and $V_{max}$ for amine removal in perfused rabbit lung

| *Amine* | n | $K_m$ *(μM)* | $V_{max}$ (nmol/lung per min) |
|---|---|---|---|
| Noradrenaline | 5 | 3.2 ± 1.1 | 7.6 ± 2.3 |
| Octopamine | 4 | 8.1 ± 1.3 | 22.6 ± 2.2 |
| Dopamine | 4 | 6.6 ± 0.6 | 19.4 ± 1.1 |
| Tyramine | 4 | 9.8 ± 0.8 | 30.0 ± 1.7 |

$n$ = number of individual determinations.

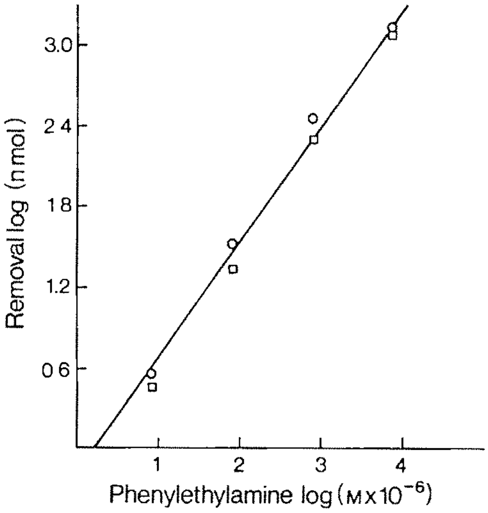

**Figure 5** Removal of phenylethylamine in the absence (O) and in the presence (□) of pargyline (10 μM) and semicarbazide (10 mM).

As shown in Figure 4, removal of phenylethylamine was linear over the relatively narrow range (0.41–3.3 μM) of concentrations used. Phenylethylamine was chosen for additional study since it was much more rapidly and extensively degraded by the perfused lung than the other amines used (see Figure 2). In separate series of experiments in which lungs were perfused with concentrations of phenylethylamine ranging from 0.83 μM to 830 μM, removal was also linear as shown in the log-log plot in Figure 5. At concentrations below 8.3 μM, 76–80% of the radioactivity appearing in fractions three, four and five (collected between 90 and 180 s after beginning perfusion) was deaminated product. The percentage of deaminated product appearing in the effluent was greatly decreased when phenylethylamine was perfused at 83 μM (49 ± 0.7%; $n = 6$) and 830 μM

($10 \pm 0.9\%$; $n=6$), suggesting that MAO is likely to be saturated at these concentrations. These results are consistent with data obtained *in vitro* which indicate that the $K_m$ for phenylethylamine deamination by lung mitochondrial MAO is approximately 7 μM (Roth & Gillis, 1974).

In view of the extensive intrapulmonary degradation of phenylethylamine, it was of interest to determine whether inhibition of such metabolism affected initial rate of removal. Therefore, lungs were perfused for 15 min with pargyline (10 μM) and semicarbazide (10 mM) and then with phenylethylamine (0.83–830 μM) and the inhibitors. These concentrations of pargyline and semicarbazide were shown previously (Roth & Gillis, 1975) to inhibit completely the metabolism of phenylethylamine by rabbit lung. Data from these experiments also are presented in Figure 5; it is evident that removal was unaffected by this treatment.

## Discussion

The process of amine removal, within the context of experiments involving perfused lung, has been defined (Gillis, 1976) as a reduction in the concentration of amine in lung effluent, when compared to that perfused through lung. Removal thus may, and in many instances does, include a process of transport from the vascular space (as in the case of NA and 5-HT) as well as subsequent metabolic degradation. However, intrapulmonary binding is not a significant component of removal in the case of biogenic amines and structurally related compounds. The biogenic amines and their metabolites leave the lung rapidly (Hughes *et al.*, 1969; Junod, 1972; Nicholas, Strum, Angelo & Junod, 1974). Transport of NA and 5-HT from the vascular space is considered to be the rate-limiting step in overall removal (Alabaster & Bakhle, 1970, 1973; Junod, 1972; Gillis & Iwasawa, 1972). Since little intracellular binding occurs, the rate of loss of amine during constant infusions would, in theory, eventually reach that of inward transport from the vasculature; at this point net removal would be zero. It is apparent, however, from this as well as earlier studies that there is substantial net removal of each amine tested; such removal must ultimately therefore reflect catabolism of the substance perfused and perhaps passive diffusion across endothelial cell membranes.

This is well illustrated by the present experiments with phenylethylamine. It can be seen (Figures 4 and 5) that phenylethylamine is removed to the greatest extent of any of the amines tested. However, over 90% (Figure 2) and at least 75% on the average, of radioactivity in lung effluent is in the form of metabolite. Accordingly, removal in this case seems to reflect largely metabolic degradation. Yet, metabolic inhibition with pargyline and semicarbazide (Figure 5) does not affect total removal. This observation, coupled with the linear relationship between a wide range of phenylethylamine concentrations and removal (Figure 5) suggests the existence of a purely passive process which brings this amine into contact with the forms of MAO responsible for its degradation (Roth & Gillis, 1975).

Noradrenaline represents the opposite extreme. In this case removal is the lowest of any amine used, but seems to involve energy-dependent, temperature and drug-sensitive transport (Bakhle & Vane, 1974; Gillis, 1976). Furthermore, metabolic inhibition does not affect net removal during short-term perfusion (Gillis & Iwasawa, 1972; Alabaster & Bakhle, 1970, 1973; Nicholas *et al.*, 1974).

The ability of rabbit lung to remove phenylethylamine and its structural analogues appears to be related to the hydrophobicity of these amines. Thus phenylethylamine, being the most liphophilic of these compounds, is removed to the greatest degree whereas, NA, with three hydroxyl groups, is removed to the least extent (Figures 2 and 4). Also, in conformity with this explanation, tyramine is more effectively removed than is octopamine or dopamine, while the latter two dihydroxylated amines are removed by lung to a greater extent than is NA. This suggests that for the more lipid soluble amines, passive transport in lung endothelium may play a major role in their removal.

The ability of the perfused rabbit lung to degrade phenylethylamine and structurally related amines is related to both uptake and the form or forms of amine oxidase which are responsible for their deamination. The three amines, phenylethylamine, tyramine and dopamine, which, in relation to percentage removal, are degraded to the greatest extent (Figure 2) are each metabolized by two or more forms of the oxidase. For example, phenylethylamine has been shown to be deaminated by the B form of the mitochondrial oxidase as well as the 'plasma' amine oxidase (Roth & Gillis, 1975; Bakhle & Youdim, 1976). Tyramine and dopamine are degraded by all three forms of amine oxidase identified in the lung vasculature (Neff & Yang, 1974). In contrast, 5-HT, octopamine and NA are deaminated by only the A form of the mitochondrial oxidase (Neff & Yang, 1974), although 5-HT is also reported to be a poor substrate for 'plasma' MAO (McEwen, Cullen & Sober, 1966).

It is noteworthy that we consistently observed metabolism of dopamine by the perfused lung. If the dopamine metabolite is biologically inactive this would imply that there is some loss of dopamine 'activity' during passage through lung. Available information on this possibility is conflicting. Thus, Boileau, Crexells & Biron (1972) found that the pressor effect of dopamine was unaffected by transpulmonary passage in rat, dog or man. However, Nicholas *et al.*

(1974) showed that dopamine was not concentrated by rat lung after a 2 min perfusion of the amine, which could well be explained by lack of binding and rapid metabolism of dopamine removed from the vascular space.

We are grateful to Miss K. Baker for her careful technical assistance. This work was supported by Grants HL 13315, HL 05942, HL 17124 and MH 26354 from the U.S. Public Health Service.

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*(Received August 2, 1976.*
*Revised October 22, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 591–597

# THE EFFECT OF SYSTEMICALLY AND TOPICALLY APPLIED DRUGS ON ULTRAVIOLET-INDUCED ERYTHEMA IN THE RAT

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1 Exposure of the skin of rats to u.v. light (>295 nm) for 30 s or longer elicited a delayed erythema response, the rate of onset increasing with the period of irradiation. The erythema was still present at 24 h and was replaced by scab formation in 48 hours.

2 Both topically applied steroidal and non-steroidal anti-inflammatory drugs reduced the erythema formation when administered immediately after u.v. exposure. Propyl gallate, an antioxidant with sun screening properties in man, also possessed topical anti-erythemic activity.

3 Both steroidal and non-steroidal anti-inflammatory drugs, systemically administered 1 h before u.v. exposure, reduced the erythema. However, the steroidal compounds were less effective than the non-steroids and reduced the intensity of erythema by less than 50%. Antagonists of 5-hydroxytryptamine (5-HT) reduced the erythema but several other drugs with different pharmacological activities were ineffective.

4 Neither topical nor systemic treatments of any of the drugs examined suppressed the scab formation at 48 hours.

5 These results and those using other selective blocking agents indicate that in the mediation of the erythema reaction prostaglandins may play a major role and 5-HT perhaps a minor one but that $H_1$ histamine receptors and $\alpha$- and $\beta$-adrenoceptors have no significant role.

## Introduction

The production of erythema in the skin of guinea-pigs exposed to ultraviolet (u.v.) light was described by Wilhelmi (1949; 1950) and has been used as a model of inflammation for the assessment of new anti-inflammatory drugs (Swingle, 1974). Furthermore, Winder, Wax, Burr, Been & Rosiere (1958) demonstrated a good correlation between the erythema-delaying action of systemically administered non-steroidal agents and their anti-rheumatic properties. However, steroidal anti-inflammatory drugs were ineffective in altering this erythema response when administered systemically (Winder *et al.*, 1958), or topically (Lambelin, Vassart-Thys & Roba, 1970).

A similar erythema response in rats has not previously been described although Logan & Wilhelm (1966a) demonstrated biphasic vascular permeability changes in the skin of u.v. irradiated rats in the absence of a significant hyperaemia. The present studies were undertaken to establish whether u.v.-induced erythema could be elicited in the rat using longer exposure times than those used by Logan & Wilhelm (1966a), in an attempt to develop a test for assessment of topically applied steroidal and non-steroidal anti-inflammatory drugs. The effects of a variety of other drugs have also been examined. A part of this work has been demonstrated to the British Pharmacological Society (Lewis, 1976).

## Methods

Male Wistar rats (Carworth Europe CE/CFHB) weighing between 80–100 g were used in groups of five. They were prepared for u.v. exposure as previously described by Winder *et al.* (1958) for guinea-pigs. Immediately after clipping, the left flanks were depilated with a cream preparation (Veet-O, Reckitt and Colman Toiletries). Twenty-four hours later three circular areas (6 mm diameter) on the flanks were exposed to u.v. irradiation provided by a Hanovia Model 10 Quartz Lamp fitted with a Kodak 18A glass filter, transmitting light greater than 295 nm wavelength. In preliminary experiments various exposure times were examined. At several intervals after u.v. exposure, the intensity of the erythema of each irradiated spot was assessed visually by a trained observer using the following scoring system: 0, no evident erythema; 0.5, an area of erythema not clearly defined as a circle; 1.0, a full circle of definite redness of any intensity.

Drugs administered topically were dissolved in absolute ethanol and applied in 0.2 ml volumes into an open-ended cylindrical reservoir held closely over the irradiated skin. They were applied immediately after irradiation. Control irradiated animals received an equal volume of absolute ethanol. The area of treated skin was used to hasten drying of the solution. The area of treated skin was then covered with plastic adhesive (Sleek, Smith and Nephew Ltd.). Drugs were administered systemically (either subcutaneously or orally in a volume of 1 ml/kg) in 5% mulgofen in water, 1 h before u.v. exposure. Animals dosed orally were fasted for 18 h previously. Control animals received an equal volume of 5% mulgofen.

In all drug treatment experiments the groups of animals were randomized and the assessor was unaware of the treatment schedule. Erythema was always assessed at 4 h after irradiation and in some experiments also at 1, 2, 3, 4 and 24 hours. Changes in erythema intensity after drug administration are expressed as percentages of the erythema intensity at 4 h in vehicle-treated rats. All the data were analysed by the randomization test of Siegel (1956). Topical $ID_{50}$ values were calculated after plotting lines of best fit through the data points and expressed as mg drug applied to the skin. After systemic administration the $ID_{50}$s were calculated for non-steroidal drugs and the $ID_{30}$s for steroidal drugs.

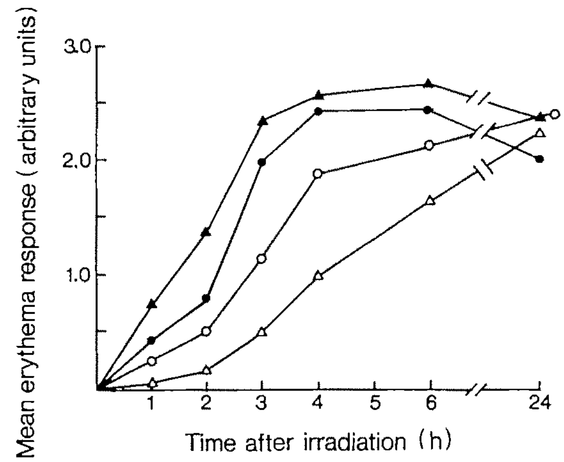

**Figure 1** The time course of erythema of rat skin after u.v. irradiation for 30 s (△), 60 s (○), 90 s (●), or 120 s (▲). Each point is the mean of 5 observations.

The following drugs were tested: aspirin, paracetamol, procaine hydrochloride (BDH), betamethasone-17-valerate (Glaxo), cyproheptadine hydrochloride, indomethacin (Merck, Sharp & Dohme), flufenamic acid (Parke Davis), fluocinolone

**Table 1** Effect of steroidal anti-inflammatory agents on u.v. erythema at 4 h (90 s exposure)

| | *Topical* | | | *Subcutaneous* | | |
|---|---|---|---|---|---|---|
| | *Dose (mg)* | *%* | *Approx $ID_{50}$ (mg)* | *Dose (mg/kg)* | *%* | *Approx $ID_{30}$ (mg/kg)* |
| Betamethasone-17-valerate | 1 | 15 | 7.8 | N/T | | |
| | 3 | 27* | | | | |
| | 10 | 55* | | | | |
| Dexamethasone | 1 | 10 | 4.8 | 0.3 | 25* | 0.5 |
| | 3 | 37* | | 1.0 | 30* | |
| | 10 | 72* | | 5.0 | 48* | |
| Fluocinolone acetonide | 1 | 30* | 4.4 | 0.3 | 13 | 1.9 |
| | 3 | 50* | | 1.0 | 26* | |
| | 10 | 58* | | 3.0 | 32* | |
| Hydrocortisone | 1 | 28 | 9.0 | 10 | 10 | >100 |
| | 3 | 40* | | 30 | 20 | |
| | 10 | 50* | | 100 | 25* | |
| Org 6216 | 0.3 | 28 | 1.0 | 10 | 21 | 30.4 |
| | 1.0 | 53* | | 30 | 33 | |
| | 3.0 | 61* | | 100 | 36* | |
| Prednisolone | 1 | 12 | 7.7 | 3 | 21 | 10.5 |
| | 3 | 45* | | 10 | 32 | |
| | 10 | 48* | | 30 | 35* | |

Drugs were applied topically immediately after u.v. exposure or subcutaneously 1 h previously. The mean % inhibition by comparison with controls and calculated ID values are shown. Groups of 10 animals were used.
N/T Not tested.
* $P < 0.05$.

acetonide, propranolol (ICI), ibuprofen and flurbiprofen (Boots), mepyramine maleate (May & Baker), methysergide bimaleate (Sandoz), phenylbutazone (Geigy), propyl gallate (Koch-Light), S.C. 19220 [1-acetyl-2(8-chloro-10,11-dihydrodibenz-(b,f)(1,4) oxazpine-10-carbonyl) hydrazine] (Searle), theophylline (Sigma), triprolidine hydrochloride (Wellcome), and other drugs synthesized by Organon Laboratories Limited. Doses refer to free base or acid.

## Results

### *Erythema formation*

Skin irradiated for 10 or 20 s showed no erythema formation within 6 hours. Responses to exposure for 30, 60, 90 and 120 s are shown in Figure 1. With 30 s exposure, erythema intensity increased progressively over a period of 24 hours. As the exposure period was increased the rate of onset of erythema was accelerated and the time taken to reach peak erythema was reduced. At 48 h all erythema responses were replaced by scabs which sloughed off after several days. For examination of drug effects an exposure period of 90 s was chosen; this produced a maximal erythema response in about 4 hours.

### *Effect of drugs on 90 s u.v. exposure*

*Topical administration.* Topically administered anti-inflammatory steroids demonstrated dose-related inhibition of the erythema intensity at 4 h, with the following order of potency: Org 6216 (11β-hydroxy-

**Table 2** The effect of various non-steroidal anti-inflammatory agents on u.v. erythema at 4 h (90 s exposure)

| | *Topical* | | | *Oral* | | |
|---|---|---|---|---|---|---|
| | *Dose (mg)* | *%* | *Approx $ID_{50}$ (mg)* | *Dose (mg/kg)* | *%* | *Approx $ID_{50}$ (mg/kg)* |
| Aspirin | 1 | 11 | | 100 | 37* | |
| | 3 | 25* | 9.2 | 200 | 59* | 148 |
| | 10 | 58* | | 300 | 68* | |
| Bufexamac | 1 | 14 | | 100 | 10 | |
| | 3 | 33* | 8.1 | 200 | 15 | >200 |
| | 10 | 53* | | | | |
| Flazalone | N/T | | | 100† | 42* | |
| | | | | 200† | 54* | 154.2 |
| | | | | 300† | 60* | |
| Flufenamic acid | 3 | 25 | | 10 | 17 | |
| | 10 | 46* | 10.9 | 30 | 46* | 38.5 |
| | 15 | 68* | | 100 | 69* | |
| Flurbiprofen | 0.1 | 22 | | | | |
| | 0.3 | 53* | 0.28 | N/T | | |
| | 1.0 | 68* | | | | |
| Ibuprofen | 0.1 | 35 | | 3 | 15 | |
| | 0.3 | 60* | 0.3 | 10 | 34* | 22.4 |
| | 1.0 | 65* | | 30 | 57* | |
| Indomethacin | 0.3 | 27 | | 0.3 | 23 | |
| | 1.0 | 46* | 1.3 | 1.0 | 47* | 1.3 |
| | 3.0 | 63* | | 3.0 | 63* | |
| Paracetamol | N/T | | | 30 | 30 | |
| | | | | 100 | 53* | 100.0 |
| | | | | 300 | 67* | |
| Phenylbutazone | 1 | 28 | | 10 | 43* | |
| | 3 | 43* | 5.6 | 30 | 59* | 17.2 |
| | 10 | 58* | | 100 | 75* | |

The drugs were applied topically immediately after u.v. irradiation or orally (or subcutaneously†) 1 h previously. The mean % inhibition by comparison with controls and the calculated $ID_{50}$ values, using groups of ten animals, are tabulated.
N/T Not tested.
* $P < 0.05$.

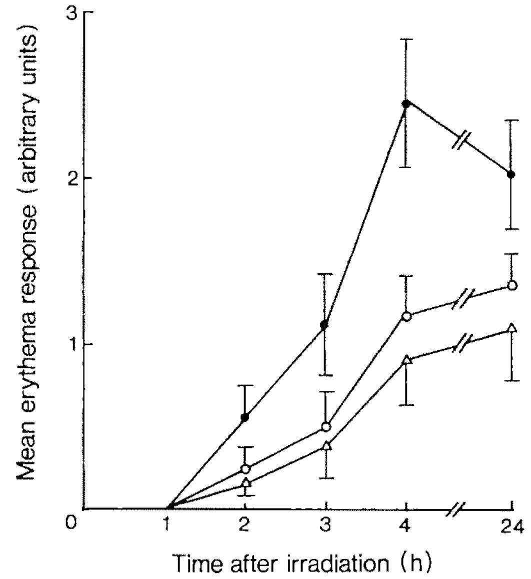

**Figure 2** The effect of topically applied drugs on the rate of development of erythema of rat skin induced by u.v. irradiation for 90 seconds. Responses in control (●) and when 3 mg of either fluocinolone acetonide (△) or phenylbutazone (○) was applied in 0.2 ml absolute ethanol immediately after irradiation are shown. Each point is the mean of 10 observations. Vertical lines show s.e. mean.

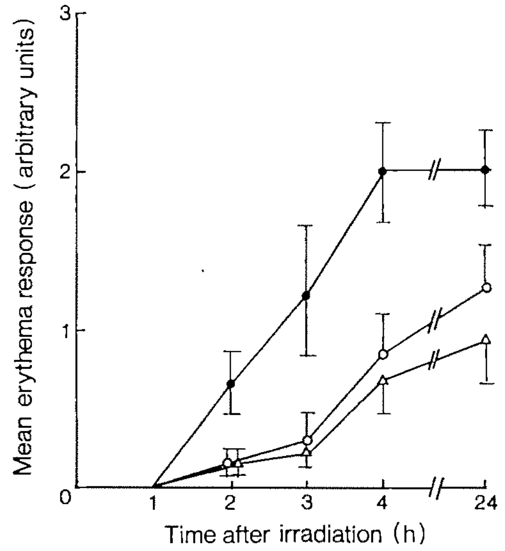

**Figure 3** The effect of systemically applied drugs on the rate of development of erythema of rat skin induced by u.v. irradiation for 90 seconds. Responses in controls (●) and in rats given fluocinolone acetonide (10 mg/kg, s.c., △) or phenylbutazone (30 mg/kg, orally, ○) 60 min before irradiation are shown. Each point is the mean of 10 observations. Vertical lines show s.e. mean.

**Table 3** Effect of various pharmacological agents on u.v. erythema at 4 h (90 s exposure)

| | *Topical* | | | *Oral* | | |
|---|---|---|---|---|---|---|
| | *Dose (mg)* | *%* | *Approx $ID_{50}$ (mg)* | *Dose (mg/kg)* | *%* | *Approx $ID_{50}$ (mg/kg)* |
| Cyproheptadine | 3 | 8 | | 3 | 0 | |
| | 15 | 12 | >15 | 10 | 6 | >15 |
| | | | | 15 | 25* | |
| Methysergide | 3 | 8 | >15 | 1 | 36* | |
| bimaleate | 15 | 7 | | 3 | 58* | 2.1 |
| | | | | 10 | 75* | |
| Mianserin | N/T | | | 1 | 19 | |
| | | | | 3 | 40* | 7.2 |
| | | | | 10 | 53* | |
| Propyl gallate | 3 | 42* | | | | |
| | 10 | 46* | 10 | N/T | | |
| | 15 | 70* | | | | |

Drugs were applied topically immediately after u.v. exposure or orally 1 h previously. The mean % inhibition by comparison with controls and calculated $ID_{50}$ values are shown. Groups of 10 animals were used.
N/T Not tested.
* $P<0.05$.

$16\alpha,17\alpha$,21-trimethyl pregna-1,4-diene-3,20-dione) > fluocinolone acetonide and dexamethasone > betamethasone-17-valerate, hydrocortisone and prednisolone (Table 1). The non-anti-inflammatory steroids, nandrolone decanoate, oestradiol, progesterone and testosterone had no significant effect in amounts of 15 mg per animal ($n = 10$).

Non-steroidal anti-inflammatories also suppressed erythema at 4 h when topically administered (Table 2). The rank order was flurbiprofen = ibuprofen > indomethacin > phenylbutazone > bufexamac $\simeq$ aspirin $\simeq$ flufenamic acid. Propyl gallate was also effective (Table 3).

Cyproheptadine and methysergide were ineffective (Table 3) as also were mepyramine, procaine, propranolol and S.C. 19220 in tests using doses of 3 and 15 mg in groups of 10 animals.

Drugs that reduced the 4 h erythema response were still effective at 24 h although scab formation at 48 h was unaffected. This is shown in Figure 2 for topically applied fluocinolone and phenylbutazone.

*Systemic administration.* None of the anti-inflammatory steroids, administered subcutaneously, inhibited erythema by more than 48%. However, they all produced dose-related inhibition, except for hydrocortisone which was ineffective at 100 mg/kg. $ID_{30}$ values were determined (Table 1).

All the non-steroidal anti-inflammatory compounds with the exception of bufexamac demonstrated dose-related inhibition of the erythema at 4 h (Table 2) when administered orally, with the following order of potencies: indomethacin > ibuprofen $\simeq$ phenylbutazone > flufenamic acid > paracetamol (4-acetamidophenol) > aspirin. Flazalone was effective subcutaneously but not examined orally.

Methysergide, mianserin and cyproheptadine administered orally reduced the erythema response (Table 3). Mepyramine (10 mg/kg), triprolidine (50 mg/kg), phenoxybenzamine (15 mg/kg), procaine (200 mg/kg), propranolol (20 mg/kg) and theophylline (20 mg/kg) were ineffective when given orally in groups of 10 rats in similar experiments.

The onset and duration of the anti-erythema effect of systemically administered drugs were similar to those described for topical administration as exemplified for fluocinolone acetonide and phenylbutazone in Figure 3. Suppression of the erythema was distinct by 2 h and effects persisted at 24 hours. Scab formation at 48 h was not influenced by either of these drugs or any other drugs that demonstrated anti-erythemic activity at 4 hours.

## Discussion

The exposure of rats to u.v. irradiation evoked an erythema in our studies but not in those of Logan & Wilhelm (1966a) who used shorter exposure periods (10 to 30 seconds).

Non-steroidal anti-inflammatory drugs are effective against u.v.-induced erythema in guinea-pigs both systemically (Winder *et al.*, 1958; Adams & Cobb, 1963; Gupta & Levy, 1973) and topically (Graeme, Peters, Maiorana & Cooper, 1975). We have shown a similar effect with these drugs in the rat. Relative effectiveness by the two routes often differed, as expected in view of differences in transport mechanisms and opportunity of inactivation. By topical application, the most potent non-steroidal drugs, ibuprofen and flurbiprofen, were more effective than the most potent steroids. Bufexamac, a drug with reported clinical topical anti-inflammatory efficacy (Lambelin *et al.*, 1970) exhibited anti-erythemic activity in this rat u.v. model.

The topical anti-erythemic activity of propyl gallate, an anti-oxidant, is of interest. In these experiments the drug was applied immediately after irradiation whereas its previously reported protective effect against u.v. irradiation in man (Kahn & Curry, 1974) and the guinea-pig (McDonald-Gibson & Schneider, 1974) could be attributed to radiation absorption.

Systemic pretreatment of irradiated rats with the non-steroidal drugs delayed the onset of erythema. Flazalone, which has been previously demonstrated to be ineffective against the u.v. reaction in the guinea-pig (Draper, Petracek, Klohs, McClure, Levy & Re, 1972), was weakly effective in the rat. The anti-erythemic activity of paracetamol was unexpected since it has been considered devoid of anti-inflammatory properties (Adams, 1960) although it was demonstrated to inhibit the u.v.-induced vascular permeability changes occurring in the mouse (Sim, 1965).

Steroidal anti-inflammatories have been reported to be inactive in the guinea-pig model when administered either topically (Lambelin *et al.*, 1970) or systemically (Winder *et al.*, 1958; Gupta & Levy, 1973). However, applied topically some were as effective as non-steroidal agents in suppressing erythema in the rat. Org 6216, a new non-halogenated steroid with local activity and lacking major systemic and local side effects (Fox, Lewis, Rae, Sim & Woods, 1977) was the most potent steroid examined. None of the non-anti-inflammatory steroids tested was effective after topical application. The steroidal anti-inflammatory drugs were less effective when administered systemically.

The relevance for man of the effects in the rat u.v. model of the steroidal anti-inflammatories is uncertain but topically applied anti-inflammatory corticosteroids can suppress u.v. erythema induced in man (Chanin, 1973; Kaidey & Kurban, 1976; Lewis & Law, unpublished observations); these substances may be of some use in the suppression of human sunburn reactions.

Neither topical nor systemic administration of either non-steroidal or steroidal compounds eliminated the erythema reaction in the rat. This parallels the finding in guinea-pigs when these agents were given systemically (Gupta & Levy, 1973) and those in man where topical and intradermal corticosteroids and topical indomethacin were examined (Kaidey & Kurban, 1976). The residual effects may be associated with necrotic changes in the skin. No major changes in rat skin histology were detected 4 h after a 90 s irradiation but by 24 h degeneration of the epidermis was marked and a large number of inflammatory cells (mainly monocytes, fibroblasts and some neutrophilic leukocytes) had accumulated in the upper dermal region (unpublished observations).

It is possible to formulate a number of theories for mechanisms of anti-erythemic effect.

The erythema induced in the rat was suppressed by the systemic administration of the 5-hydroxytryptamine (5-HT) antagonists, methysergide and mianserin, but not by the antihistaminics, mepyramine and triprolidine. Cyproheptadine, a drug which is both a 5-HT antagonist and histamine antagonist, showed only a weak effect. Bonta (1969) demonstrated a potent anti-erythemic activity of methysergide in the guinea-pig u.v. erythema model and speculated that its mechanism of action could be a result of both its anti-5-HT and its vaso-constrictor effects. In view of the possible dual mode of action of methysergide and possibly other 5-HT antagonists, it is not certain that 5-HT is a mediator of u.v. erythema in the rat.

The early phase of the biphasic vascular permeability changes that occurred after u.v. irradiation in the rat has also been shown to be susceptible to the 5-HT antagonist bromlysergic acid diethylamide (BOL) (Logan & Wilhelm, 1966b). However, only the early phase (5–15 min) of increased vascular permeability appeared to be reduced by BOL. The response corresponding to the erythema production (4 h) was only slightly suppressed by this 5-HT antagonist.

There is mounting evidence that prostaglandins may play a role in the mediation of u.v.-induced erythema. After u.v. irradiation prostaglandin formation occurs in human (Greaves & Sondergaard, 1970; Mathur & Ghandi, 1972), rat (Mathur & Ghandi, 1972) and guinea-pig skin (Sekura Snyder, 1976). Moreover, non-steroidal anti-inflammatory drugs are able to inhibit prostaglandin formation in the skin (Greaves & McDonald-Gibson, 1973) and recently Sekura Snyder (1976) has demonstrated that topical application of indomethacin reduces both the erythema and prostaglandin levels present in the u.v. irradiated guinea-pig skin. There is also evidence that corticosteroids may inhibit synthesis and the release of these substances (Greaves & McDonald-Gibson, 1972; Lewis & Piper, 1975). Propyl gallate at high concentration also inhibits prostaglandin formation (van Dorp, 1967) a factor which may contribute to its topical anti-erythemic efficacy. However, the prostaglandin antagonist, S.C. 19220, was not effective against the erythema although when applied topically it reduced the oedema response to croton oil in the rat (Sanner, 1974).

Ultraviolet damage to the skin causes lysosomal membrane rupture and consequent hydrolytic enzyme release (Johnson & Daniels, 1969; Honigsmann, Wolff & Konrad, 1974). Both steroidal and non-steroidal anti-inflammatory drugs exhibit lysosomal membrane-stabilizing properties (Weissman & Fell, 1962; Ignarro, 1971) that may reduce enzyme release from the lysosomes. Such an effect may play a role in their inhibition of u.v. erythema.

The cutaneous vasoconstrictor activity of the steroids and possibly of certain of the non-steroids, particularly when they are applied topically, may also contribute to suppression of the u.v. erythema. However, steroids with potent vasoconstrictor actions on the cutaneous vasculature in man, such as betamethasone-17-valerate, are not the most potent anti-erythemic compounds in the rat; neither are steroids with high anti-erythemic activity, such as Org 6216, the most active vasoconstrictor agents (Fox *et al.*, 1977).

In conclusion, the mechanism of u.v.-induced erythema induction in the rat and the mode of action of drugs that reduce this response is uncertain, but 5-HT release, prostaglandin formation, lysosomal enzyme release and/or possibly local vasoconstrictor activity may all be contributory factors.

We would like to thank Miss Dawn Bell, Miss Rona Mason, Mrs Linda Patterson and Mrs Ann Wilmot for their excellent technical assistance during these studies.

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*(Received August 4, 1976.*
*Revised December 1, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 599–601

# EFFECTS OF $\Delta^9$-TETRAHYDROCANNABINOL AND CANNABIDIOL ON A $Mg^{2+}$-ATPase OF SYNAPTIC VESICLES PREPARED FROM RAT CEREBRAL CORTEX

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1 $\Delta^9$-Tetrahydrocannabinol and cannabidiol both exhibited a concentration-related inhibition of $Mg^{2+}$-ATPase of vesicles prepared from synaptosomes isolated from rat cerebral cortex. Cannabidiol was about 3 times more potent than tetrahydrocannabinol.

2 These results were similar to those obtained previously using drugs with well established anticonvulsant activity.

3 Tetrahydrocannabinol at a sub-inhibitory concentration (1 μM) increased the activity of the $Mg^{2+}$-ATPase relative to values obtained with vehicle controls.

## Introduction

The effects of a wide range of drugs on the activity of the magnesium-activated adenosine triphosphatase ($Mg^{2+}$-ATPase) of synaptic vesicles prepared from rat cerebral cortex have previously been studied (Gilbert & Wyllie, 1975; 1976). Of the drugs tested only those known to have anticonvulsant activity were found to inhibit this ATPase. $\Delta^9$-Tetrahydrocannabinol (tetrahydrocannabinol) and cannabidiol, both constituents of cannabis, exhibit anticonvulsant activity in rats (Izquierdo, Orsingher & Berardi, 1973) and mice (Karler, Cely & Turkanis, 1973) and it was therefore of interest to investigate the effect of these cannabinoids on the activity of the vesicular $Mg^{2+}$-ATPase.

## Methods

Mitochondrial and synaptosomal fractions were prepared from cerebral cortices of male Sprague–Dawley rats (body wt. 150 to 350 g) by a modification of the method of Gray & Whittaker (1962) as described by Gilbert & Wyllie (1976). Synaptic vesicles were prepared by osmotic disruption of synaptosomes and subsequent sucrose density gradient centrifugation as described by Whittaker, Michaelson & Kirkland (1964). The fractions were stored at −23°C.

For ATPase assays, vesicle-containing fractions were resuspended in 50 mM imidazole/HCl buffer pH 7.4 containing $MgCl_2$ (5 mM). The assays were carried out with 0.9 ml samples of this mixture, each sample containing 0.1 to 0.2 mg protein.

The samples were pre-incubated for 15 min at 37°C before the reactions were started by addition of 0.1 ml Tris-ATP solution (4 mM final concentration). Sodium dodecyl sulphate (DDS 1 ml, 0.8% w/v in water) was used to stop the reactions after 10 minutes. The phosphate contents of the clear solutions were determined by the method of Bonting, Simon & Hawkins (1961). Blanks were prepared by addition of DDS after the pre-incubation period, before the addition of the ATP.

The activity of $Mg^{2+}$-ATPase was calculated by subtraction of the phosphate released in the blank from that in the $Mg^{2+}$-ATPase reaction mixture.

The protein contents of solutions were determined by the method of Lowry, Rosenbrough, Farr & Randall (1951).

Drugs were added to the pre-incubation medium in aliquots of 5 μl. Tetrahydrocannabinol and cannabidiol were added in absolute ethanol. Appropriate control experiments with ethanol were included. Drug effects were expressed as the percentage change in the activity of the enzyme in the absence of ethanol or any other drug. Limits of error were expressed as standard error and differences between means were evaluated by Student's *t* test (paired).

## Results

The results are summarized in Table 1. Ethanol (78 mM) produced a small but statistically significant degree of inhibition of the $Mg^{2+}$-ATPase. Cannabidiol also inhibited the enzyme. This inhibition was significantly greater than ethanol control values at

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concentrations of 1 μM and above. The effect of tetrahydrocannabinol appeared to be biphasic. At concentrations ranging upwards from 3 μM tetrahydrocannabinol was inhibitory, but at a drug concentration of 1 μM the activity of the vesicular $Mg^{2+}$-ATPase was 14.5 ± 1.4% greater than it was in the presence of ethanol alone. The degree of inhibition produced by both cannabinoids reached a maximum at and above concentrations of 0.1 mM. This maximum effect consisted of a 69 to 70% inhibition of the $Mg^{2+}$-ATPase activity. Experiments with sub-maximal concentrations of the cannabinoids showed the inhibitory effect of the vesicular $Mg^{2+}$-ATPase to be concentration-related and the relative inhibitory potency of cannabidiol and tetrahydrocannabinol to be about 3.

Because of the low solubility of the cannabinoids in water, the question as to whether or not inhibition of vesicular $Mg^{2+}$-ATPase by ethanol and by the cannabinoids is additive was not investigated. Such an investigation was however carried out with the more water soluble phenytoin. In the absence of ethanol, phenytoin inhibited the ATPase by 36.5 ± 1.1% at a concentration of 1 μM and by 71.3 ± 0.5% at a concentration of 20 μM. In the presence of ethanol, inhibition by these concentrations of phenytoin was respectively 49.5 ± 3.7% and 78.7 ± 0.6%. In these experiments, ethanol produced an inhibition of 8.6 ± 2.3%. The results confirm that phenytoin can inhibit vesicular $Mg^{2+}$-ATPase and suggest that the inhibitory effects of phenytoin and of ethanol are indeed additive.

## Discussion

The results show that tetrahydrocannabinol and cannabidiol can both inhibit vesicular $Mg^{2+}$-ATPase. Since both drugs have been reported to exhibit anticonvulsant activity *in vivo* (Izquierdo *et al.*, 1973; Karler *et al.*, 1973) the results add to the evidence (Gilbert & Wyllie, 1976) for the concept that the capacity to inhibit the vesicular $Mg^{2+}$-ATPase is a characteristic property of anticonvulsant drugs.

There are similarities between the effects of the cannabinoids reported in this paper and the effects (reported elsewhere, Gilbert & Wyllie, 1976) of other anticonvulsant drugs. The concentrations at which tetrahydrocannabinol and cannabidiol inhibited vesicular $Mg^{2+}$-ATPase are of the same order as those at which other anticonvulsant drugs produce inhibition. As with the other anticonvulsants so far studied, the inhibition of vesicular $Mg^{2+}$-ATPase produced by the cannabinoids at maximal concentrations was not complete. This incomplete inhibition may result from the heterogeneity of the nerve terminal content of the synaptosome preparation. This point has been discussed elsewhere (Gilbert & Wyllie, 1976).

A sub-inhibitory concentration of tetrahydrocannabinol was found to increase $Mg^{2+}$-ATPase activity relative to values observed in the presence of ethanol, the vehicle in which the drug was dissolved. Further experiments are needed to determine whether this observation reflects direct activation of the enzyme by tetrahydrocannabinol or antagonism of the inhibitory effect of ethanol. In addition it would be interesting to determine whether, at sub-inhibitory concentrations, cannabidiol increases the activity of $Mg^{2+}$-ATPase.

Finally, it is of interest that the relative potency of tetrahydrocannabinol and cannabidiol in inhibiting vesicular $Mg^{2+}$-ATPase is of about the same value as the relative potency of these cannabinoids in decreasing the susceptibility of rat hippocampus to seizure discharges caused by afferent stimulation (Izquierdo *et al.*, 1973). However, without further experimental data it would not be profitable to speculate about this correlation which could well have arisen by chance and the question of whether there is a 'cause-effect' relationship between the inhibition of vesicular $Mg^{2+}$-ATPase and anticonvulsant activity still remains to be answered.

We thank Dr M.C. Braude of NIMH for supplies through the MRC of $\Delta^9$-tetrahydrocannabinol and Professor R. Mechoulam for a gift of cannabidiol.

**Table 1** Effects of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBD) on vesicular $Mg^{2+}$-ATPase

| | *Mean % Inhibition of $Mg^{2+}$-ATPase (± s.e.)* | | | | |
|---|---|---|---|---|---|
| *Cannabinoid concentration (μM)* | *$\Delta^9$THC* | P | *CBD* | P | *Ethanol control* |
| 1 | 3.8 ± 1.9 (4) | <0.01 | 19.3 ± 2.1 (4) | <0.02 | 10.7 ± 2.4 (4) |
| 3 | 19.3 ± 2.6 (2) | <0.02 | 39.6 ± 2.1 (2) | <0.09 | 8.6 ± 2.3 (2) |
| 10 | 43.2 ± 1.4 (4) | <0.001 | 57.5 ± 1.7 (4) | <0.001 | 10.7 ± 2.4 (4) |
| 100 | 68.8 ± 4.3 (5) | <0.001 | 65.1 ± 1.2 (5) | <0.001 | 11.8 ± 2.0 (5) |
| 1000 | 67.7 ± 0.5 (2) | <0.06 | 69.6 ± 0.6 (2) | <0.06 | 12.9 ± 4.4 (2) |

Number of experiments are given in parentheses. Student's *t* test (paired) was used to evaluate (*P*) differences between drug treatment and ethanol controls. A typical value for vesicular $Mg^{2+}$-ATPase activity (in the absence of ethanol) was 5.25 ± 0.17 (4) μmol Pi mg protein$^{-1}$ h$^{-1}$.

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*(Received August 11, 1976.*
*Revised October 12, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 603–614

# EFFECTS OF SYMPATHETIC INNERVATION AND TEMPERATURE ON THE PROPERTIES OF RAT HEART ADRENOCEPTORS

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1 The pharmacological characteristics of adrenoceptors at different temperatures were assessed on the basis of the effects of various $\alpha$- and $\beta$-adrenoceptor agonists and antagonists on electrically-driven left atria and spontaneously-beating pairs of atria from rats.

2 Phenoxybenzamine (Pbz) potentiated inotropic responses of left atria to noradrenaline (NA) at 31°C, produced significantly less potentiation at 24°C and inhibited responses at 17°C; it had little effect on responses to $CaCl_2$. Both Pbz and phentolamine inhibited responses to phenylephrine more effectively at 17 than at 31°C. *N*-cyclohexylmethyl-*N*-ethyl-$\beta$-chloroethylamine hydrochloride (GD-131), a haloalkylamine with negligible $\alpha$-adrenoceptor blocking activity, caused only potentiation of responses to NA at 17°C.

3 The presence of phentolamine during incubation with Pbz eliminated block of responses to NA and revealed a potentiation that was equivalent at all three temperatures tested. Phentolamine did not alter the block of responses to 5-hydroxytryptamine by Pbz. Protection of $\alpha$-adrenoceptors by phentolamine during exposure to [$^3$H]-Pbz significantly decreased the amount of label bound to the myocardium at 17°C, but did not alter binding at 31°C.

4 Inhibition of responses to NA by propranolol decreased with temperature, and the magnitude of the change increased with the concentration of propranolol. Compared to 31°C, the effect of the highest concentration of propranolol. (4.0 μM) was significantly decreased at 24°C, and the effects of all except the lowest concentration (0.04 μM) were significantly decreased at 17°C.

5 The potency of isoprenaline decreased and that of phenylephrine increased at low temperatures, and their potency ratio was much lower at 17 than at 31°C for both the inotropic and chronotropic responses of spontaneously-beating atria. However, the ratio was unaffected by temperature in electrically-driven left atria. A similar difference between spontaneously-beating and driven preparations is apparent in the data of other workers, but its basis is not clear.

6 Atria from rats pretreated with 6-hydroxydopamine (6-OHDA) were sensitized to the effects of NA, and there was no increase in $\alpha$-adrenoceptor properties at low temperatures. Little $\alpha$-adrenoceptor activity could be demonstrated in chemically denervated atria at any temperature. 6-OHDA pretreatment did not alter the binding of [$^3$H]-Pbz at 31°C, but decreased it significantly at 17°C. Pretreatment with reserpine caused some sensitization, but did not significantly alter the characteristics of the adrenoceptors or their responses to temperature.

7 It is concluded that the adrenoceptors of rat atria are affected by temperature in much the same way as those of frog hearts, although the transition from $\beta$- to $\alpha$-adrenoceptor properties may begin at a slightly higher temperature. $\alpha$-Adrenoceptor properties appear to require the presence of intact adrenergic nerves but not the presence of normal stores of neurotransmitter.

## Introduction

Several studies have shown that the adrenoceptors mediating inotropic and chronotropic responses of frog hearts are $\beta$ at higher and have a major $\alpha$-component at lower temperatures (Kunos & Szentiványi, 1968; Buckley & Jordan, 1970; Kunos, Yong & Nickerson, 1973b, Harri, 1973; Tirri, Harri & Laitinen, 1974). The observations that reciprocal changes in the blocking effectiveness and the tissue retention of $\alpha$- and $\beta$-adrenoceptor antagonists occurred sharply between 17 and 23°C, and that alkylation of $\alpha$-adrenoceptors by phenoxybenzamine at a low temperature prevented the appearance of $\beta$-adrenoceptors when the temperature was raised were interpreted to indicate that $\alpha$- and $\beta$-adrenoceptors may be interconvertible forms of a single basic structure (Kunos & Nickerson, 1976). Recent studies have suggested that adrenoceptors of the mammalian myocardium are similarly affected by temperature (Kunos, Vermes-Kunos, Boyd & Nickerson, 1973a; Benfey, Kunos & Nickerson, 1974; Amer & Byrne, 1975). The present observations on rat atria confirm the shift from $\beta$- towards $\alpha$-adrenoceptor properties at low temperatures, and indicate that the $\alpha$-adrenoceptor component in inotropic responses requires intact sympathetic innervation. An abstract of some of these findings has been published (Kunos *et al.*, 1973a).

## Methods

### *Preparation of isolated atria and experimental design*

Male Sprague-Dawley rats (180–300 g) were lightly anaesthetized with ether and the hearts quickly removed. Left atria or spontaneously-beating pairs of atria were isolated and mounted in 20 ml organ baths containing modified Krebs-Henseleit solution of the following composition (mM): NaCl 115.3, KCl 4.6, $CaCl_2$ 1.8, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, $KH_2PO_4$ 1.1 and glucose 11.1, (pH 7.43) bubbled with 5% $CO_2$ and 95% $O_2$. The temperature was controlled by a refrigerated, constant temperature circulating water bath (Lauda, Model K-2-R). Left atria were driven through small platinum electrodes by square-wave pulses of 3 ms duration at a voltage slightly above threshold (0.3–1.5 V) and a rate of 1 Hz (Grass stimulator, Model S88). Isometric contractions were recorded by force-displacement transducers (Grass, FT 83C) and a polygraph (Grass, Model 7). Rates of contraction were determined directly from the polygraph records.

The preparations were allowed to equilibrate for at least an hour at 31°C with frequent changes of the medium and the temperature was then set at 31, 24 or 17°C. Each preparation was used at only one temperature. Agonists were added cumulatively and the maximal response was determined in every experiment. The basal contractile amplitude often decreased significantly after the first concentration-response curve was determined. In such cases, a second concentration-response curve to the same agonist was obtained and used as control. All responses are expressed as a percentage of the maximal control increase in force of contraction or rate. Absolute values for force of contraction are also indicated for most series of experiments. The basal contractile amplitude increased and the absolute change in force in response to inotropic drugs decreased with decreasing temperature. Some preparations were insensitive to all agonists at 17°C, and remained insensitive despite extensive washing and repeated challenges with agonist. These are not included in the calculations of mean responses.

Atria treated with phenoxybenzamine (Pbz) or [$^3$H]-phenoxybenzamine ([$^3$H]-Pbz) were exposed to the drug (0.7–7.3 μM) freshly diluted every 10 min from a stock solution in propylene glycol containing 0.01N HCl. After a 40 min exposure, the atria were washed with drug-free medium for 60–120 min and responses to the agonist retested. Preparations exposed to [$^3$H]-Pbz were then removed for counting retained radioactivity. Exposure to propranolol was for 40 min and responses to noradrenaline (NA) were retested in the presence of the antagonist. Atria were first exposed to a concentration of 0.04 μM propranolol. After retesting with NA and washout of the drugs, the same preparations were exposed to concentrations of 0.4 and 4.0 μM propranolol in sequence, and responses to NA were retested at the end of each 40 min incubation period. The effects of phentolamine (2.6 μM) on inotropic responses to phenylephrine were also tested after a 40 min exposure with the antagonist still in the bath.

### *Receptor protection experiments*

Left atria were exposed to phentolamine (26.5 μM) at 17, 24 or 31°C. After 10 min, [$^3$H]-Pbz (7.3 μM) was added to the medium, which was replaced every 10 min by fresh solution containing both drugs. After a 40 min exposure to both drugs, the preparations were washed for at least an hour to remove phentolamine and all unreacted [$^3$H]-Pbz. Responses to NA were then retested and the preparations removed for counting radioactivity.

### *Pretreatment with 6-hydroxydopamine (6-OHDA) and reserpine*

Rats with an initial body weight of 80–100 g were pretreated with 6-OHDA according to the schedule proposed by Thoenen & Tranzer (1968). Two doses of 50 mg/kg were given intravenously within 24 h, followed a week later by 2 x 100 mg/kg. Experiments on isolated left atria were performed one week after the last dose of 6-OHDA. This dose schedule has been shown to reduce the NA content of rat hearts by more than 90%, and the resulting supersensitivity is qualitatively the same as that after other forms of sympathetic denervation (Haeusler, Haefely & Thoenen, 1969).

To deplete NA stores without destruction of sympathetic nerve endings, rats were pretreated with reserpine. Two treatment schedules were used: a single dose of 5.0 mg/kg given intraperitoneally 24 h before the experiment, or 0.5 mg/kg given daily for 7 days. Results with atria from rats on the two treatment schedules were almost identical and were pooled.

### *Determination of retained radioactivity*

Atria exposed to [$^3$H]-Pbz were washed for 2 h, including the test period with an agonist, before freeze-drying. The dry tissue was weighed and digested in 0.5 ml of NCS tissue solubilizer (Amersham/Searle) at 50°C; 14.5 ml of counting solution containing 4.0 g of 2,5-diphenyloxazole (PPO) and 0.05 g of 2-*p*-phenylene-bis(5-phenyloxazole) (POPOP) in 1.0 litre of reagent grade toluene was added. Radioactivity was measured in a liquid scintillation counter (Nuclear Chicago, Mark I), using the channels ratio technique and a quench calibration curve. Counting efficiency was in the range of 20–32%. Radioactivity is expressed as concentration of labelled drug in the dry tissue.

### *Drugs*

$[^3H]$-phenoxybenzamine hydrochloride was prepared by the method of Nikawitz, Gump, Kerwin & Ullyot (1952), as detailed elsewhere (Yong & Nickerson, 1973). Other drugs used were phenoxybenzamine hydrochloride (Smith Kline & French), (–)-noradrenaline ((–)-arterenol bitartrate, K & K), (–)-phenylephrine hydrochloride (K & K), isoprenaline ((±)-isopropylnoradrenaline hydrochloride, K & K), phentolamine (Rogetine methanesulphonate, Ciba), (±)-propranolol (Inderal, Ayerst), 5-hydroxytryptamine (5-HT, serotonin creatinine sulphate hydrate, Calbiochem), 6-hydroxydopamine hydrobromide (Regis), reserpine (Serpasil, Ciba), and GD-131 (*N*-cyclohexylmethyl - *N* - ethyl - *β*- chloroethylamine hydrochloride, synthesized by Dr M.S. Yong). Most of the drugs were freshly diluted before each experiment in 0.9% w/v NaCl solution (saline) containing 0.01 N HCl. Concentrations are expressed as M in the bath.

### *Statistics*

Differences between means were evaluated by Student's two-tailed *t* test for unpaired data or the *t* test for paired data, as appropriate, and differences with a *P* value of 0.05 or less were considered significant. Mean values are presented with their standard errors.

## Results

### *Effects of α-adrenoceptor antagonists on inotropic responses to noradrenaline and phenylephrine at different temperatures*

At all temperatures tested (17, 24 and 31°C), the baseline contractile amplitudes of electrically-driven left atria were transiently increased by Pbz (0.7–7.3 μM), but declined during the exposure period and were equal to or slightly less than the control values after washout of the drug. Pbz (7.3 μM) potentiated inotropic responses to noradrenaline (NA) at 31°C; concentration-response curves were shifted to the left (log dose-ratio –0.99 ± 0.12), but the maximal developed tension was unaltered (Figure 1a). A similar exposure to Pbz at 17°C inhibited responses to NA; the change in the mean $EC_{50}$ was not statistically significant, but the maximal response was significantly decreased ($P < 0.02$) (Figure 1c). A concentration of 0.7 μM Pbz had similar but smaller effects. Although the effect of Pbz at 24°C was similar to that at 31°C, the shift to the left of the NA concentration-response curve was significantly smaller (log dose-ratio –0.53 ± 0.10, $P < 0.02$) and the maximal developed tension was slightly reduced (Figure 1b). Inotropic responses to phenylephrine were inhibited by Pbz at both 17 and 31°C, but the block was significantly greater at the low than at the high temperature (Figure 2a & c). A similar temperature-dependent change in the blocking effect of phentolamine (2.6 μM) was also noted (Figure 2b & d). The basal amplitude was not significantly altered by phentolamine at either temperature but, unex-

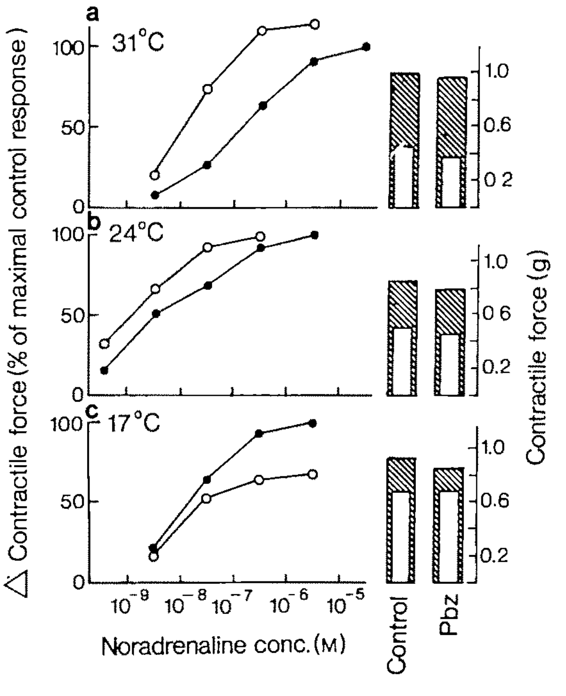

**Figure 1** Effects of phenoxybenzamine (Pbz) on inotropic responses of rat left atria to noradrenaline (NA) at different temperatures. Control concentration-response curves (●); after 40 min exposure to Pbz (7.3 μM) (○). Open columns show basal contractile force and hatched columns the maximal force in response to NA. Number of experiments (a) 14 (31°C); (b) 8 (24°C); (c) 15 (17°C).

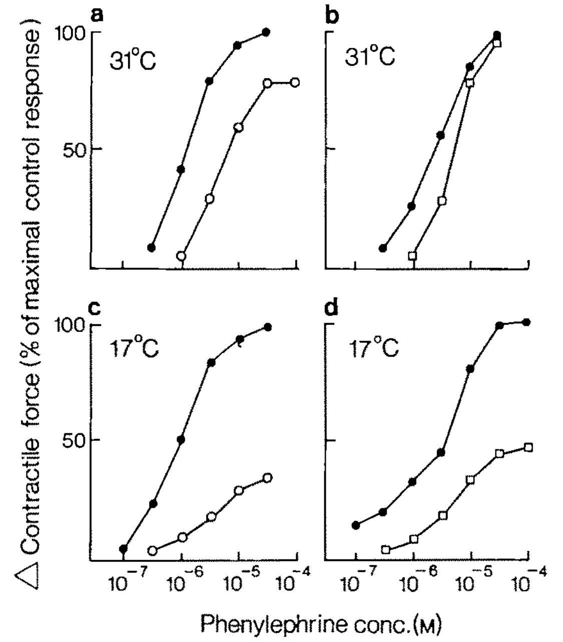

**Figure 2** Inhibition of inotropic responses to phenylephrine by phenoxybenzamine (Pbz) in (a) and (c) and by phentolamine in (b) and (d) at 17 and 31°C. Control concentration-response curves (●); after exposure to Pbz (7.3 μM) (○); in the presence of phentolamine (2.6 μM) (□). Number of experiments: (a) 6; (b) 4; (c) 5; (d) 4.

pectedly, the maximal response to phenylephrine at 17°C was significantly decreased.

As observed in frog hearts, the Pbz blockade at 17°C developed slowly during the washout period and the first NA concentration-response curve often showed some potentiation. Blockade then developed gradually and was stable after 1 hour. Figure 3 shows that almost complete block of the inotropic response of a left atrial preparation to NA at 17°C was associated with only a slight decrease in the contractile response to $CaCl_2$. In three experiments at 17°C, atria were exposed for 40 min to 10.4 μM GD-131, a haloalkylamine with negligible α-adrenoceptor blocking properties. This drug moderately potentiated inotropic responses to NA in all experiments.

### *Effects of phentolamine on block by and binding of [³H]-phenoxybenzamine in rat left atria*

Exposure in the presence of phentolamine (26.5 μM) abolished the temperature dependence of the effects of Pbz (7.3 μM), and potentiation of response to NA at all three temperatures (Figure 4) was similar to that produced by Pbz alone at 31°C. This indicated that inhibition of responses to NA by Pbz at 17°C and the decrease in potentiation at 24°C were due to α-adrenoceptor blockade and that the potentiating effect of Pbz is largely independent of temperature. In three experiments at 17°C, Pbz (7.3 μM) almost completely blocked inotropic responses to 5-hydroxytryptamine (5-HT), and this effect was unaltered by exposure in the presence of phentolamine (26.5 μM) in three other preparations.

In contrast to frog hearts (Kunos & Nickerson, 1976), the radioactivity retained by rat atria exposed to [³H]-Pbz was not significantly changed by temperature, and was even slightly higher at 31°C than at the lower temperatures (Figure 5, open columns). However, protection against Pbz block by phentolamine at 17°C was associated with a

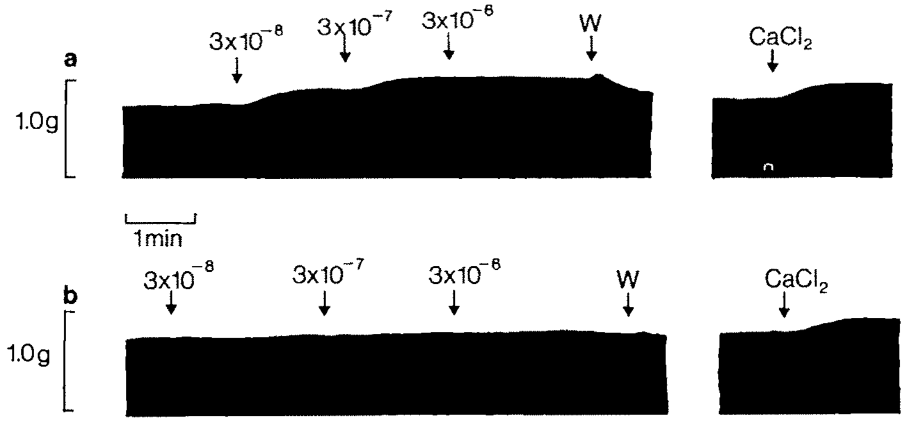

**Figure 3** Block of inotropic responses to noradrenaline (NA) by [³H]-phenoxybenzamine (7.3 μM) without significant inhibition of the response to added $CaCl_2$ (2.25mM) in electrically-driven rat left atrium at 17°C. Stimulation: 1 Hz, 3 ms, 1.25 V. (a) Control; (b) 60 min after washout of phenoxybenzamine. Indicated concentrations of NA (M) administered at arrows. W indicates wash.

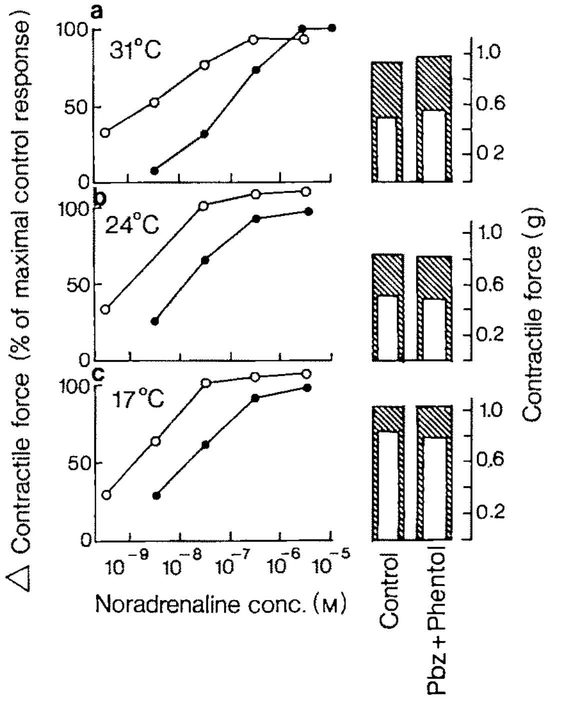

**Figure 4** Effects of exposure to [$^3$H]-phenoxybenzamine ([$^3$H]-Pbz) in the presence of phentolamine on inotropic responses of rat left atria to noradrenaline (NA) at different temperatures. Control concentration-response curves (●); after exposure to [$^3$H]-Pbz (7.3 μM) + phentolamine (26.5 μM) (○). Open columns show basal contractile force and hatched columns the maximal force in response to NA. Number of experiments: (a) 14 (31°C); (b) 8 (24°C); (c) 15 (17°C).

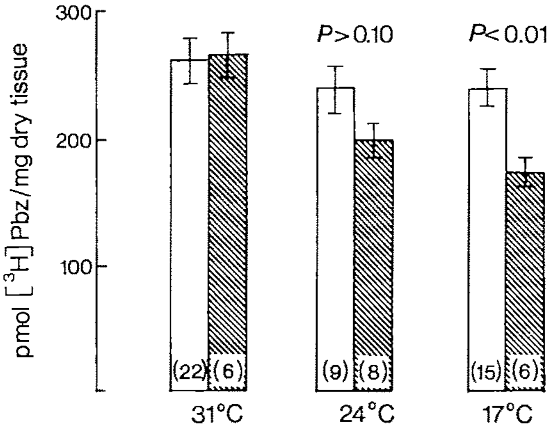

**Figure 5** Retention of radioactivity by rat left atria after exposure to [$^3$H]-phenoxybenzamine ([$^3$H]-Pbz) (7.3 μM) alone (open columns) or in the presence of phentolamine (26.5 μM) (hatched columns). Numbers of experiments are shown in parentheses. Vertical lines show standard errors of means.

significant decrease in retained radioactivity, whereas at 31°C phentolamine did not alter the amount of label retained (hatched columns). At 24°C phentolamine caused an appreciable, but not statistically significant decrease in binding.

### *Effects of propranolol on inotropic responses of left atria to noradrenaline at different temperatures*

The effects of propranolol on inotropic responses to NA at 17, 24 and 31°C are shown in Table 1. The inhibitory effects of all except the lowest concentration (0.04 μM) decreased significantly with decreasing temperature, and this change was apparent at 24 as well as at 17°C with the highest concentration of propranolol (4.0 μM).

**Table 1** Effects of propranolol on inotropic responses of electrically-driven left atria to noradrenaline

| | *Propranolol concentration (μM)* | | | |
|---|---|---|---|---|
| | *0* | *0.04* | *0.4* | *4.0* |
| *Temp.* | *$pD_2$** | *Log dose-ratios†* | | |
| 31°C<br>(8) | 7.40±0.16 | 0.76±0.11 | 1.92±0.25 | 2.99±0.27 |
| 24°C<br>(7) | 7.83±0.10 | 0.69±0.06 | 1.54±0.07 | 2.05±0.09** |
| 17°C<br>(5) | 7.66±0.13 | 0.72±0.14 | 1.27±0.16* | 1.82±0.12** |

* Means and standard errors of negative log of noradrenaline $EC_{50}$'s. † Means and standard errors of the log of the ratio of noradrenaline $EC_{50}$'s in the presence and in the absence of propranolol.
Asterisks indicate significance of difference from corresponding value at 31°C (*$P<0.05$; **$P<0.01$). Numbers in parentheses show number of experiments.

### *Sensitivity of atria to adrenoceptor agonists at different temperatures*

The $pD_2$ values (negative logarithm of the molar $EC_{50}$) for the inotropic effects of isoprenaline and phenylephrine were determined from control concentration-response curves of spontaneously-beating pairs of atria and of electrically-driven left atria. $pD_2$ values for chronotropic responses were also determined in the former preparations. Sensitivity to isoprenaline was significantly lower and that to phenylephrine significantly higher at 17 than at 31°C for both the inotropic and chronotropic responses of spontaneously-beating preparations (Table 2). This resulted in a considerable decrease in the potency ratio of the two amines at the lower temperature. However, in electrically-driven left atria the inotropic potencies of both amines increased with decreasing temperature, and there was no change in the potency ratio.

### *Responses of left atria from rats pretreated with 6-hydroxydopamine*

Haloalkylamines can inhibit the uptake of NA into adrenergic nerve endings (Iversen, Salt & Wilson, 1972), which could contribute to the potentiation of myocardial responses to NA by these drugs. The receptor protection experiments presented above showed that the potentiating effect of Pbz is present at all temperatures, but is masked by its $\alpha$-adrenoceptor blocking action at 17°C. Therefore, destruction of sympathetic nerve endings by 6-OHDA might reduce potentiation and reveal the blocking action of Pbz more clearly.

Pretreatment of rats with 6-OHDA significantly increased the sensitivity of left atria to NA. The mean $EC_{50}$ was decreased more at 17 than at 31°C, 42- vs. 20-fold; responses to phenylephrine were potentiated much less. In contrast to control preparations, exposure to Pbz had no initial inotropic effect in atria from 6-OHDA pretreated rats, which probably reflected depletion of endogenous NA. Exposure to [$^3$H]-Pbz (7.3 $\mu$M) at 31°C only slightly potentiated inotropic responses to NA and the somewhat greater maximal tension increase was probably related to the slightly lower baseline amplitude after Pbz (Figure 6a). Unexpectedly, Pbz did not significantly alter the NA concentration-response curve of atria from pretreated animals at 17°C. The blocking action observed in control preparations at this temperature was completely absent (Figure 6b). Pbz blockade of responses to phenylephrine at both temperatures (see Figure 2) was also nearly abolished by 6-OHDA pretreatment.

The amount of radioactivity retained after exposure to [$^3$H]-Pbz (7.3 $\mu$M) at 31°C was almost identical in atria from 6-OHDA pretreated and control animals (Figure 7). However, the absence of $\alpha$-adrenoceptor blockade at 17°C in the atria from 6-OHDA pretreated rats was associated with a significantly lower retention of label than that of control atria exposed at the same temperature. This was analogous to the reduction in binding observed when $\alpha$-adrenoceptors were protected from [$^3$H]-Pbz block by

**Table 2** Sensitivity of spontaneously-beating pairs and electrically-driven left atria to isoprenaline and phenylephrine at different temperatures

| | *Temp.* | *Isoprenaline* | *Phenylephrine* | *Log potency-ratio* |
|---|---|---|---|---|
| *Left atria*<br>(inotropic) | 31°C | 9.29 $\pm$ 0.12$^a$<br>(34) | 5.50 $\pm$ 0.06<br>(24) | 3.79 |
| | | ** | * | |
| | 17°C | 9.78 $\pm$ 0.11<br>(12) | 5.99 $\pm$ 0.23<br>(10) | 3.79 |
| *Pairs of atria*<br>(a) Inotropic | 31°C | 9.69 $\pm$ 0.22<br>(6) | 5.52 $\pm$ 0.10<br>(5) | 4.17 |
| | | * | ** | |
| | 17°C | 8.80 $\pm$ 0.28<br>(9) | 5.94 $\pm$ 0.09<br>(10) | 2.86 |
| (b) Chronotropic | 31°C | 9.49 $\pm$ 0.21<br>(11) | 5.64 $\pm$ 0.09<br>(16) | 3.85 |
| | | ** | | |
| | 17°C | 8.55 $\pm$ 0.24<br>(10) | 5.87 $\pm$ 0.10<br>(10) | 2.73 |

[a] Means and standard errors of $pD_2$'s.
Asterisks indicate significance of difference between values at the two temperatures (*$P < 0.05$; **$P < 0.01$).
Numbers in parentheses show numbers of experiments.

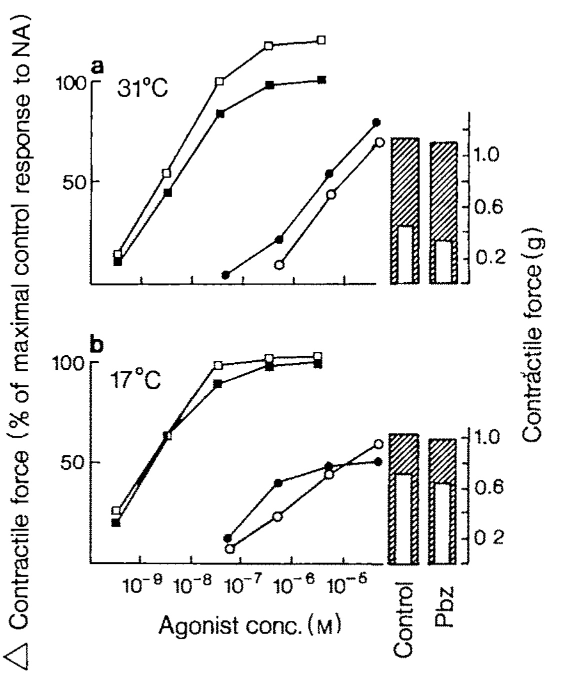

**Figure 6** Effects of [$^3$H]-phenoxybenzamine ([$^3$H]-Pbz) on inotropic responses of left atria from rats pretreated with 6-hydroxydopamine to noradrenaline (NA) and phenylephrine at 17 and 31°C. Control concentration-response curves for NA (■) and for phenylephrine (●); after [$^3$H]-Pbz (7.3 μM), NA (□) and phenylephrine (○). Open columns show basal contractile force and hatched columns the maximal force in response to NA. Number of experiments: (a) 10 (31°C); (b) 9 (17°C).

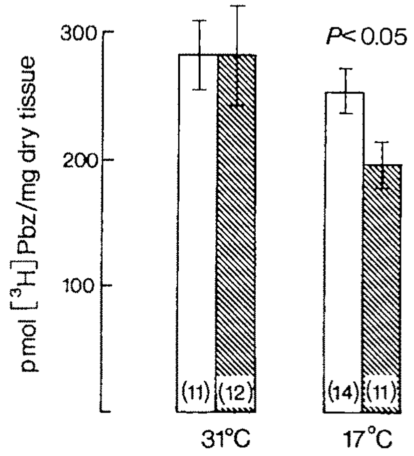

**Figure 7** Retention of radioactivity by left atria from control (open columns) and 6-hydroxydopamine pretreated (hatched columns) rats after exposure to [$^3$H]-phenoxybenzamine ([$^3$H]-Pbz) (7.3 μM). Numbers of experiments are shown in parentheses. Vertical lines show standard errors of means.

phentolamine (see Figure 5).

Pretreatment with 6-OHDA also abolished the decrease in the effect of propranolol noted when the temperature of control preparations was reduced. Cumulative doses of propranolol caused slightly greater shifts to the right of the NA concentration-response curves of 6-OHDA pretreated (Table 3) than those of control preparations (Table 1) at 31°C. However, the propranolol block was at least as great at 17 as at 31°C, significantly greater for 0.4 μM, and was clearly greater for all concentrations than that produced in control atria at 17°C. Thus, the inotropic effect of NA in atria from 6-OHDA pretreated rats appeared to be mediated only by β-adrenoceptors at both test temperatures.

### *Responses of left atria from rats pretreated with reserpine*

The results with atria from 6-OHDA pretreated rats indicated that adrenergic innervation may be necessary for the shift from β- to α-adrenoceptor properties in rat atria at low temperature. This effect of innervation might be due to the neurotransmitter or to some other influence of the nerves. A tentative differentiation of these possibilities was sought by testing atria from rats pretreated with reserpine to deplete endogenous NA. Reserpine pretreatment moderately increased sensitivity of left atria to both

**Table 3** Effects of propranolol on inotropic responses to noradrenaline in electrically-driven left atria from rats pretreated with 6-hydroxydopamine

| | *Propranolol concentration (μM)* | | | |
|---|---|---|---|---|
| | *0* | *0.04* | *0.4* | *4.0* |
| *Temp.* | *$pD_2$* | | *Log dose-ratios* | |
| 31°C | 8.76±0.26 | 1.07±0.24 | 2.08±0.18 | — |
| 17°C | 9.51±0.24 | 1.65±0.33 | 2.97±0.26* | 3.83±0.30 |

* Significantly different ($P<0.05$) from ratio at 31°C. Four experiments at each temperature.

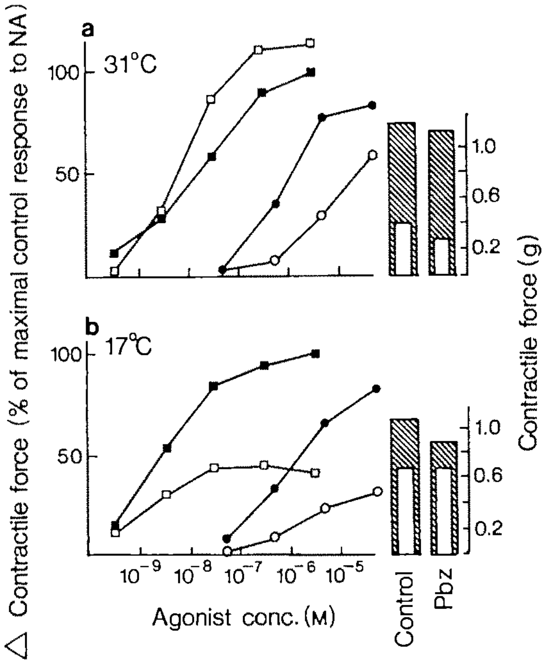

**Figure 8** Effects of phenoxybenzamine (Pbz) on inotropic responses of left atria from rats pretreated with reserpine to noradrenaline (NA) and phenylephrine at 17 and 31°C. Control concentration-response curves for NA (■) and for phenylephrine (●), after Pbz (7.3 μM), NA (□) and phenylephrine (○). Open columns show basal contractile force and hatched columns the maximal force in response to NA. Number of experiments: (a) 5 (31°C); (b) 13 (17°C).

NA and phenylephrine, but did not alter the temperature-dependence of Pbz blockade (Figure 8). Pbz (7.3 μM) potentiated the inotropic response to NA (log dose-ratio −0.45 ± 0.18) and partially blocked that to phenylephrine at 31°C. The same exposure to Pbz at 17°C inhibited responses to NA, and blocked responses to phenylephrine more effectively than at the higher temperature. At both temperatures, the block produced by Pbz was more pronounced, and the potentiation at 31°C was significantly smaller than in atria from control animals (see Figures 1 and 2). These results indicate that the α-adrenoceptor component of the inotropic response to sympathomimetic amines at 31°C and the increase in this component when the temperature is reduced to 17°C are not abolished and tend to be enhanced by depletion of endogenous NA.

## Discussion

The experimental results presented above are in agreement with and extend previous observations made on frog isolated hearts (Kunos & Szentiványi, 1968; Buckley & Jordan, 1970; Kunos *et al.*, 1973b; Harri, 1973; Kunos & Nickerson, 1976). The observed changes in the effectiveness of drugs acting on α- and β-adrenoceptors can be interpreted to indicate an interconversion of β- to α-adrenoceptors as the temperature of a mammalian heart preparation was lowered. In both frog and rat preparations the greatest change in adrenoceptor properties occurred below 24°C, but the upper limit of receptor transition appears to be somewhat higher in the mammalian heart. In the present experiments on rat atria there was an increase in the effect of α- and a decrease in the effect of β-adrenoceptor antagonists when the temperature was reduced from 31 to 24°C.

Pbz is known to react with nonspecific sites (Harvey & Nickerson, 1954) as well as with several receptors, and the change in its effectiveness with temperature has been attributed to the former (Benfey, 1975). However, several observations strongly support the interpretation that inhibition of inotropic responses to sympathomimetics by Pbz at low temperatures is due to specific block of α-adrenoceptors: (1) The change in the effectiveness of Pbz with temperature is in the wrong direction to be due to nonspecific alkylation of tissue constituents. Formation of the intermediate aziridinium ion and, particularly, its subsequent, rate-limiting reaction with nucleophilic groups increases rapidly with temperature (Harvey & Nickerson, 1953; Rosen & Ehrenpreis, 1972). As expected from this change in chemical reactivity, we have observed that the nonspecific binding of [$^3$H]-Pbz to frog heart homogenates increases progressively with incubation temperature, more than 10-fold over the range of 4 to 24°C (Kunos & Nickerson, unpublished results). In contrast, the block of inotropic responses of rat atria was greater at low temperatures. (2) The change in block with temperature was essentially the same for the competitive antagonist phentolamine as it was for Pbz. (3) The presence of phentolamine during incubation with Pbz at 17°C prevented block of responses to both NA and phenylephrine, but did not alter the potentiation of responses to NA at 31°C. Prevention of the block by phentolamine strongly supports the involvement of specific α-adrenoceptors because the same concentration of this competitive α-adrenoceptor antagonist had no effect on the block of responses to 5-HT by Pbz. (4) GD-131, a haloalkylamine that has been shown to lack significant α-adrenoceptor blocking activity in other preparations (Nickerson & Gump, 1949; Kalsner & Nickerson, 1969), but which has a nonspecific reactivity comparable to Pbz (Harvey & Nickerson, 1954), potentiated rather than blocked responses to NA at 17°C. (5) Pbz effectively inhibited responses to NA at 17°C but had a minimal effect on those to $CaCl_2$. This indicated that the block was not due to the inhibition of calcium fluxes that can be produced by high concentrations of a haloalkylamine (see Triggle, 1972). (6) Block by Pbz was largely eliminated by pretreatment with 6-OHDA. It is difficult to see how exposure to this drug one and two weeks prior to the experiment could alter nonspecific alkylation of the myocardium.

Observations on the tissue retention of [$^3$H]-Pbz also indicate an increase in the number of $\alpha$-adrenoceptors at low temperatures. In contrast to our previous observations on frog hearts (Kunos & Nickerson, 1976), the total retention of label was not increased at low temperatures. However, effective protection of $\alpha$-adrenoceptors by phentolamine significantly reduced the binding of [$^3$H]-Pbz at 17°C, whereas phentolamine had little effect on either binding of or potentiation by [$^3$H]-Pbz at 31°C. This indicated that, as in the frog heart, the binding of Pbz to $\alpha$-adrenoceptors was increased at the low temperature to an identifiable fraction of the label. It is probable that failure to demonstrate a significant difference in the total radioactivity retained by rat atria at 31 and 17°C was due to greater nonspecific binding at the higher temperature. The reason for this difference in nonspecific binding in the two preparations is not clear, but it could be related to the fact that the experimental conditions (route of access of drugs, temperature) were more physiological for frog hearts than for rat atria. The presence of cut surfaces in rat left atria might also promote the type of temperature-dependent, nonspecific binding of Pbz that we have observed in frog heart homogenates (see above).

The results with antagonists provided the most clearcut evidence of changes in adrenoceptor characteristics. The effectiveness of both a competitive (phentolamine) and a nonequilibrium (Pbz) $\alpha$-adrenoceptor antagonist increased as the temperature was lowered, and there was a reciprocal change in the effect of the $\beta$-adrenoceptor antagonist propranol. $\alpha$-Adrenoceptor blockade did not reduce responses to NA and had only a small effect on those to phenylephrine at 31°C, as would be expected from the many observations which indicate that inotropic responses of the mammalian myocardium at physiological temperatures are mediated predominantly by $\beta$-adrenoceptors (see Furchgott, 1970). As in many other studies of $\alpha$-adrenoceptor blockade in the heart (Wenzel & Su, 1966; Govier, 1968; Nakashima, Maeda, Sekiya & Hagino, 1971; Schümann, Endoh & Wagner, 1974; Mugelli, Ledda & Mantelli, 1976), we used somewhat higher (micromolar) concentrations of antagonists than are needed in most smooth muscle preparations. However, even among smooth muscle structures, $\alpha$-adrenoceptors can vary significantly (Sheys & Green, 1972), and a considerable difference between smooth and cardiac muscle (Schümann *et al.,* 1974) is not surprising. Although their contributions are difficult to assess, other characteristics of both the drugs and the tissue could also contribute to the apparent relative resistance to blockade. The significant $\beta$-adrenoceptor activity still present at 17°C could be important in 'masking' the degree of block produced by $\alpha$-adrenoceptor antagonists. Pronethalol has been reported to reveal an $\alpha$-adrenoceptor component of the inotropic action of adrenaline and NA, but not of isoprenaline, at 32°C (Govier, 1968), and the combination of pindolol and phentolamine produces a greater than additive block of responses to phenylephrine (Schümann *et al.,* 1974). It is of interest that the concentration-response relation for propranolol is flatter at 17 than at 31°C in control atria (Table 1), but is much steeper at 17°C in atria from 6-OHDA pretreated rats (Table 3) in which the response has little or no $\alpha$-adrenoceptor component. Similarly, the slope for phentolamine block of responses to phenylephrine at 37°C was very low, but was converted to that expected of a competitive antagonist by a low concentration of pindolol (Schümann *et al.,* 1974).

Occult potentiation could also reduce the apparent block of responses to NA by Pbz at 17°C; protection of $\alpha$-adrenoceptors by phentolamine revealed a comparable potentiation at all temperatures. The interaction of blockade and potentiation is also apparent in the observation that the significant shift to the left of the NA concentration-response curve produced by Pbz at 31°C was abolished in guinea-pig atria incubated with Pbz at 17°C and subsequently tested at the higher temperature (Benfey, 1975, Table 2). This observation also indicates that the block produced by Pbz at a low temperature persisted when the temperature was raised, as previously reported for frog hearts (Kunos *et al.,* 1973b; Kunos & Nickerson, 1976).

Irrespective of the mechanisms responsible for differences in the apparent sensitivity of different tissues to adrenoceptor blockade, dose or concentration *per se* does not establish or exclude the interaction of a drug with specific receptors. This must be assessed on the basis of the relative inhibition of responses to specific and nonspecific agonists, and in the case of nonequilibrium antagonists, particularly, by 'receptor protection' experiments (Furchgott, 1954).

Although changes in the effects of sympathomimetic amines observed in the present study are compatible with the changes in adrenoceptor characteristics deduced from the effects of antagonists, some details are difficult to explain. The receptor specificity of adrenoceptor agonists is lower than that of antagonists, but the potency ratio of an agonist with predominantly β- and one with relatively less β- and more α-adrenoceptor activity would be expected to decrease as the temperature was lowered if there is a shift from β towards α in receptor characteristics. The potency ratios of isoprenaline and phenylephrine for both inotropic and chronotropic responses did decrease considerably with temperature when the drugs were tested on spontaneously-beating preparations, but there was no change in the ratio in electrically-driven rat atria. A similar failure to demonstrate temperature-induced changes in agonist ratios in electrically-driven frog ventricle strips was recently reported (Benfey, 1975), but these results are difficult to compare with others because the preparations were much less sensitive to adrenaline and isoprenaline than are spontaneously-beating frog hearts (Erlij, Cetrangolo & Valadez, 1965; Kunos & Nickerson, 1976).

Both low temperature and electrical stimulation can influence the potency of agonists by mechanisms other than changes in receptor characteristics, and a coincidence of these effects could mask a change in receptor properties. Changes in the balance of α- and β-adrenoceptors are readily demonstrated by agonist ratios when only one of these factors is involved, i.e. temperature in spontaneously-beating preparations (Buckley & Jordan, 1970; Broadley, 1972; Harri, 1973; Tirri *et al.,* 1974; Amer & Byrne, 1975), or electrical driving at a high temperature in atria from rats with altered thyroid state (Nakashima *et al.,* 1971; Kunos *et al.,* 1974).

Factors other than changes in receptor characteristics that could affect agonist potency ratios include the release of endogenous NA by electrical stimulation (Jewell & Blinks, 1968), and the effects of released transmitter could be augmented by reduced reuptake (Kirpekar & Wakade, 1968) and decreased catechol-*O*-methyltransferase (COMT) activity (Opperman, Ryan & Haavik, 1972; Munoz-Ramirez, Ryan & Buckner, 1975) at lower temperatures. Any change in COMT activity could also have a direct effect on the isoprenaline-phenylephrine potency ratio. Some involvement of released endogenous NA is suggested by our observation that reserpine pretreatment increased the apparent α-adrenoceptor characteristics of electrically-driven rat atria, but a full explanation of differences between the responses of electrically-driven and spontaneously-beating preparations to agonists must await further studies.

The fact that shifts in receptor characteristics are more readily revealed by antagonists than by agonists could be due to some action of the former that would 'direct' unstable, transitional phase receptors into a specific configuration. There is no direct evidence for this mechanism at present, but it might provide an explanation for the unexpected reduction by phentolamine of maximal responses to phenylephrine. This was a consistent finding, but it cannot be explained on the basis of current receptor theory. Such an action of antagonists might also be the basis for the observations that very low concentrations of propranolol, present during incubation with Pbz and subsequently washed out, greatly increase the block produced by a standard exposure to Pbz in frog hearts at low temperature (Kunos & Nickerson, unpublished observations), and decrease the threshold blocking concentration of Pbz in atria from hypothyroid rats (Kunos, 1977)

Our experiments on atria from 6-OHDA pretreated rats revealed an unexpected effect of sympathetic innervation on receptor characteristics. In addition to supersensitivity to the inotropic effect of NA, atria from pretreated rats showed almost pure β-adrenoceptor characteristics under all conditions tested. Pbz did not inhibit responses to NA at 17°C, and propranolol was even more effective at this temperature than at 31°C. In addition, block of responses to phenylephrine by Pbz was markedly reduced at both temperatures, which indicated that even the small α-adrenoceptor component of responses at 31°C was decreased by denervation. Similarly, denervation did not alter the binding of [$^3$H]-Pbz at 31°C, but significantly less label was retained by denervated than by control atria at 17°C. These results all indicate that the α-adrenoceptor component of inotropic responses requires intact sympathetic innervation and that denervation 'stabilizes' adrenoceptors in the β configuration. A similar decreased effectiveness of α-adrenoceptor antagonists on chronically denervated cat nictitating membranes has been observed, and has been attributed to qualitative changes in the α-adrenoceptors (Varma, 1966) or to other postsynaptic changes (Langer & Trendelenburg, 1968).

Depletion of the NA content of rat atria by reserpine pretreatment produced moderate supersensitivity, but did not prevent the changes in adrenoceptor properties induced by temperature. Indeed, Pbz blocked responses to NA at 17°C even more effectively than it did in control atria. These observations suggest that adrenergic innervation may affect adrenoceptor characteristics by a mechanism independent of the presence of neurotransmitter. The nature of this effect is unknown, but passage of neuroplasmic constituents other than acetylcholine across the myoneural junction has been shown in skeletal muscle (Korr, Wilkinson & Chornock, 1967), and the possibility that such substances are involved in various 'neurotrophic' phenomena has been considered (Guth, 1968).

The shift in the properties of myocardial adrenoceptors from β towards α after thyroidectomy was also prevented by 6-OHDA but not by reserpine pretreatment (Kunos & Mucci, 1975). This further illustrates the role of sympathetic innervation in determining adrenoceptor characteristics and the similarity of temperature- and hormone-induced adrenoceptor interconversions.

Few details of metabolic changes that may accompany denervation are known, but it appears not unreasonable to suggest that denervation supersensitivity may be associated with an increase in some component of tissue metabolism. If this is so, the observed effects of chemical denervation would fit into the general pattern relating adrenoceptor properties to tissue metabolism that is emerging from other studies. In addition to low temperature, metabolic inhibitors (Nickerson & Nomaguchi, 1950; Kunos & Szentiványi, 1968; Matheny & Ahlquist, 1975), thyroid deficiency (Nakashima *et al.*, 1971; Kunos *et al.*, 1974) and a reduced rate of contraction of ventricular muscle (Endoh & Schümann, 1975; Mugelli *et al.*, 1976) appear to promote $\alpha$-adrenoceptor properties and, conversely, muscle activity augments $\beta$-adrenoceptor characteristics (Szentiványi, Kunos & Juhász-Nagy, 1970). $\beta$-Adrenoceptors are generally more sensitive than $\alpha$ to noradrenaline ($\beta_1$) and adrenaline, and this interconversion may serve to adapt the mechanical response of a tissue to its metabolic state.

This work was supported by grants from the Medical Research Council of Canada and the Quebec Heart Foundation. We wish to thank Dr M.S. Yong for synthesis of the [$^3$H]-phenoxybenzamine and GD-131.

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*(Received August 13, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 615–620

# THE SYMPATHOMIMETIC ACTIVITY OF FENFLURAMINE HYDROCHLORIDE ON RAT VAS DEFERENS

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1 The peripheral, pharmacological effects of the anorexigenic agent, fenfluramine hydrochloride, have been investigated on rat isolated vas deferens.

2 Characteristic spiked contractions were observed within 2 to 3 min after exposure to fenfluramine; these contractions reached a rate of around 13 per min and were of variable height.

3 Pre-treatment of vasa with the indirectly acting sympathomimetic amine, tyramine, greatly reduced both the height and rate of contraction induced by fenfluramine.

4 The uptake inhibitor, desipramine, required a concentration in excess of 10 µM to affect fenfluramine-induced contractions. Effects of desipramine on fenfluramine contractions were of equal magnitude whether desipramine was administered before fenfluramine or at the height of the fenfluramine-induced contractions.

5 Pre-treatment with debrisoquine (0.5 mM), reduced the contractions in response to fenfluramine over a period of time.

6 Fenfluramine, added to vasa from rats which had been injected intraperitoneally with 5 mg/kg reserpine 24 h and 48 h previously, failed to induce its characteristic contractions.

7 It is concluded that fenfluramine can be classed as an indirectly acting sympathomimetic amine on peripheral adrenergic nerve terminals.

## Introduction

Amphetamine and fenfluramine have both been shown to produce an anorectic effect in animals and man. However, they differ pharmacologically in a number of respects, the most noticeable being the marked central locomotor stimulating properties of amphetamine (Le Douarec & Neveu, 1970).

The comparative biochemical modes of action of amphetamine and fenfluramine, in producing anorectic effects in animals and man have been well documented. Both noradrenaline (Weissman, Koe & Tenen, 1966) and dopamine (Kruk, 1973) have been suggested as intermediates in the anorectic effects of amphetamine. On the other hand, it has been suggested that the mediator for fenfluramine is 5-hydroxytryptamine (Jespersen & Scheel-Krüger, 1970; Funderburk, Hazelwood, Ruckart & Ward, 1971; Samanin, Ghezzi, Valzelli & Garattini, 1972; Clineschmidt, 1973; Ghezzi, Samanin, Bernasconi, Tognoni, Gerna & Garattini, 1973). The biochemical action of fenfluramine is not, however, confined to the serotoninergic nerve terminal, since fenfluramine has also been shown to affect noradrenaline concentrations, both centrally and peripherally (Costa, Groppetti & Revuelta, 1971; Sipes, Ziance & Buckley, 1971; Ziance, Sipes, Kinnard & Buckley, 1972; Mitchell & Mottram, 1976).

The purpose of the present study was to investigate, more fully, the *in vitro* peripheral effects of fenfluramine in an attempt to characterize the mode of action of the drug on noradrenergic nerve terminals.

## Methods

Experiments were carried out on the vasa deferentia of male Wistar rats. The rats, weighing 200–275 g, were killed by a blow to the head. The vasa were excised, stripped of extraneous material and suspended in 10 ml organ baths containing Tyrode solution (composition (g/l): NaCl 8.0, KCl 0.2, $MgCl_2$ 0.2, $CaCl_2$ 0.2, $NaH_2PO_4$ 0.05, $NaHCO_3$ 1.0 and glucose 1.0) maintained at 37°C and aerated with a mixture of 95% $O_2$ and 5% $CO_2$. Isometric contractions were recorded with Devices 2 oz strain gauge tranducers and two channel recorder. All drugs were freshly prepared and administered in Tyrode solution.

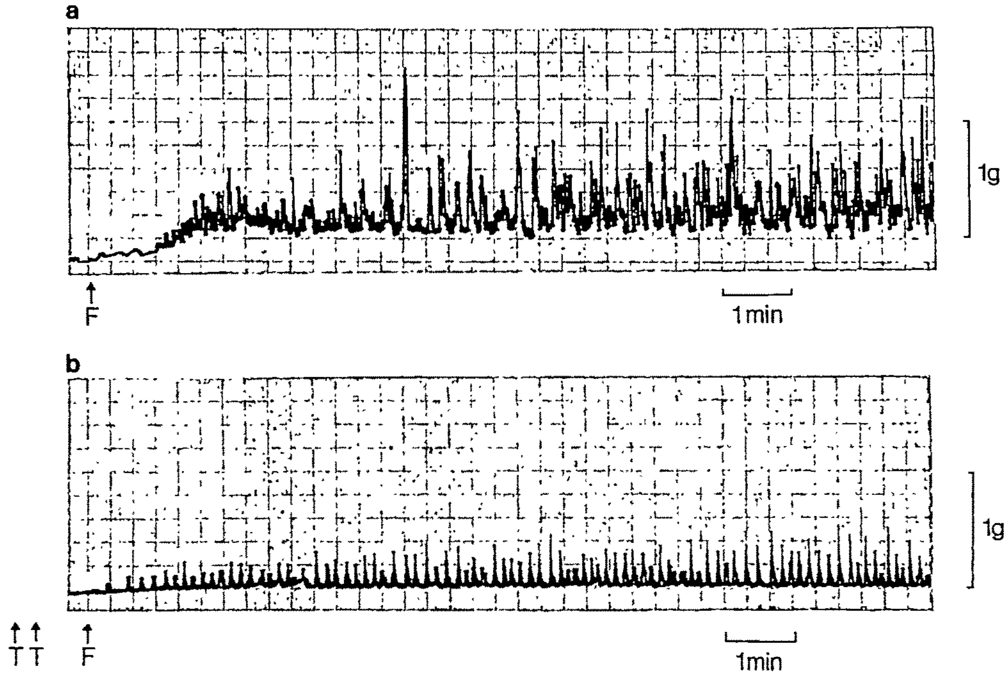

**Figure 1** Tracings from one of five experiments, showing the effects of pre-treatment with 0.4 mM tyramine (T) before the addition of 0.2 mM fenfluramine (F) to both test and control vasa deferentia of the rat. (a) Control; (b) tyramine pre-treated. The bathing fluid was changed at the end of each 3 min exposure to tyramine. Vertical calibration 1 g, horizontal calibration 1 minute.

### *Pre-treatment with tyramine*

Paired vasa, from the same animal, were set up in organ baths. The test vas was exposed to 0.4 mM tyramine, administered twice at 3 min intervals, fresh Tyrode solution being added at the end of each 3 min period; 0.2 mM fenfluramine was then added to both test and control vasa.

### *Desipramine pre- and post-treatment*

Various concentrations of desipramine (0.1 μM to 0.1 mM) were added to one of a pair of vasa, 2 min before the addition of 0.2 mM fenfluramine to both paired vasa. The same range of concentrations of desipramine was also added to vasa at the height of the contractions produced by 0.2 mM fenfluramine.

### *Pre-treatment with debrisoquine*

Debrisoquine sulphate (0.5 mM) was added to one of a pair of vasa 5 min before the addition of fenfluramine (0.2 mM). The same dose of fenfluramine was added to the control vas. Contractions were recorded over a 30 min period.

### *Effects of reserpine-treatment on fenfluramine responses*

Reserpine (5 mg/kg, i.p.) was injected into rats on two consecutive days; 24 h after the second injection, animals were killed in the usual manner and their vasa suspended in organ baths. Control rats were treated similarly, but an equal volume of vehicle was injected. Fenfluramine (0.2 mM) was then added to the vasa from both test and control rats.

The following drugs were used: fenfluramine hydrochloride (Ponderax), tyramine hydrochloride, desipramine hydrochloride, debrisoquine sulphate, reserpine, noradrenaline acid tartrate.

## Results

### *Pre-treatment with tyramine*

Contractions of control vasa defentia were recorded after the addition of 0.2 mM fenfluramine hydrochloride (Figure 1). Contractions began within 2 min and reached a rate of around 13 contractions per minute. The heights of contraction varied, but reached maximum 20 to 30 min after the addition of fenfluramine.

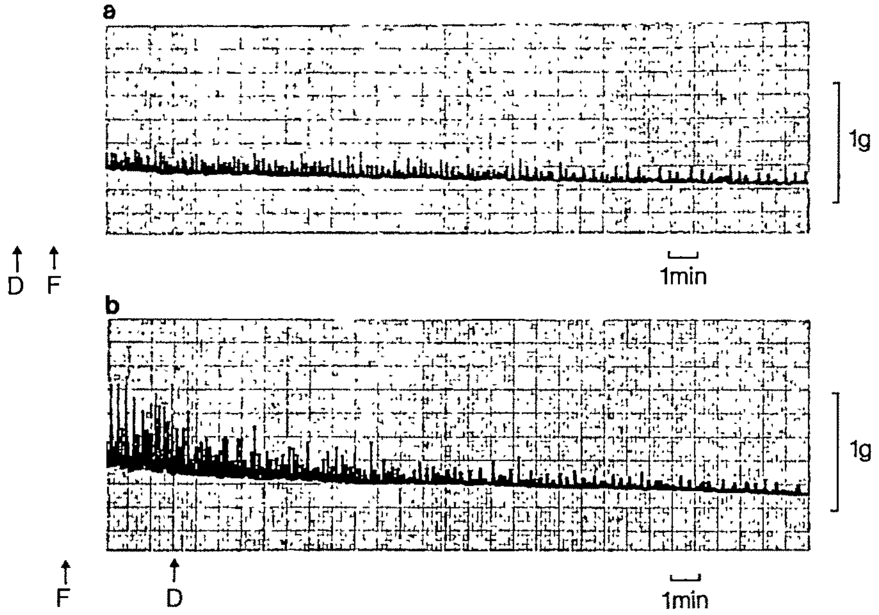

**Figure 2** Tracings from the results of experiments ($n=22$) on the effect of pre- and post-treatment with desipramine, on the contractile response of rat vas deferens to fenfluramine. (a) Contractions of a vas deferens that had received 50 μM desipramine (D) 2 min before the addition of 0.2 mM fenfluramine (F). (b) Effects of 50 μM desipramine (D) added to the vas deferens at the height of the contractions produced by 0.2 mM fenfluramine (F). Vertical calibration 1 g, horizontal calibration 1 minute.

Pre-treatment with the indirectly acting sympathomimetic amine, tyramine, before the addition of fenfluramine, caused a reduction in both the rate and height of the contractions produced by fenfluramine (Figure 1).

### *Desipramine pre- and post-treatment*

Concentrations of desipramine between 0.1 μM and 10 μM, administered to vasa before the addition of fenfluramine, did not affect the subsequent rate or height of contraction produced by fenfluramine. Concentrations of desipramine in excess of this (20 μM, 50 μM and 0.1 mM) reduced the height, and subsequently the rate of contraction produced by fenfluramine (Figure 2).

In addition to this, the same range of concentrations of desipramine were added to vasa at the height of their contractions to fenfluramine, and a parallel effect was seen. No reduction in rate or height of contraction was produced until a dose of 10 μM desipramine was exceeded, after which a similar effect to that produced by pre-treatment of desipramine was observed (Figure 2).

### *Pre-treatment with debrisoquine*

Vasa which had been treated with debrisoquine (0.5 mM), before the addition of fenfluramine, exhibited a reduced response to fenfluramine, compared with control vasa that had received fenfluramine alone (Figure 3). It was noted that debrisoquine itself, when administered alone to the vas deferens, produced fenfluramine-like contractions, although these were soon reduced and ultimately abolished.

### *Effects of pretreatment with reserpine on responses to fenfluramine*

Fenfluramine failed to produce its characteristic contractions on vasa from animals which had received reserpine (Figure 4). These tissues were then challenged with a submaximal dose of noradrenaline, the contractions produced indicating that the tissues had maintained their sensitivity towards the neurotransmitter (Figure 4). Following the washout of noradrenaline from the organ bath containing these reserpinized tissues, which had been exposed to fenfluramine, the characteristic contractions induced

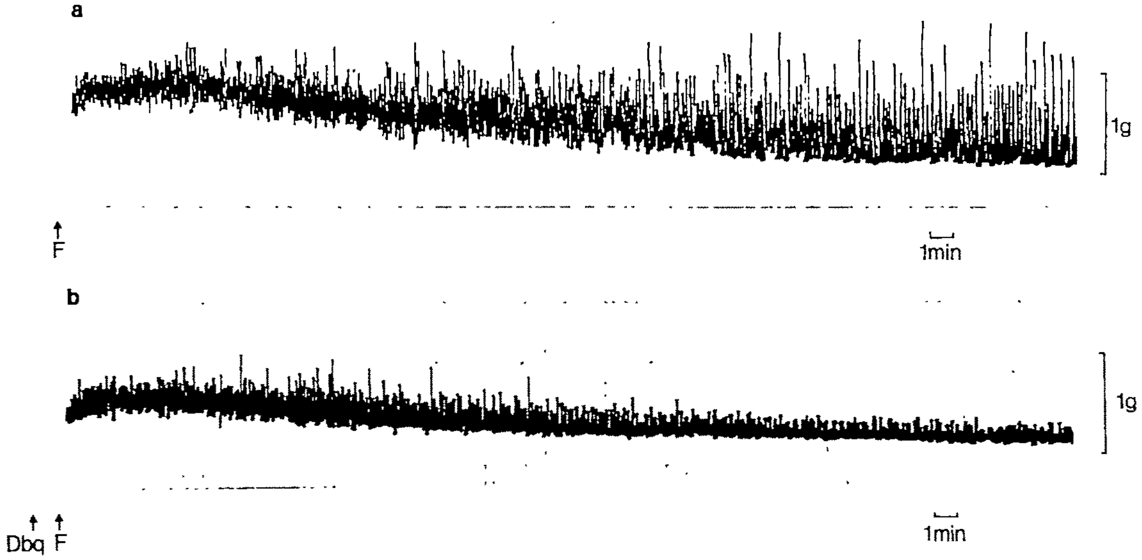

**Figure 3** Tracings from one of five experiments in which the effects of pre-treatment with 0.5 mM debrisoquine (Dbq) on contractions produced by 0.2 mM fenfluramine (F) in rat vas deferentia were observed. (a) Control; (b) debrisoquine pre-treated vas deferens. Vertical calibration 1 g, horizontal calibration 1 minute.

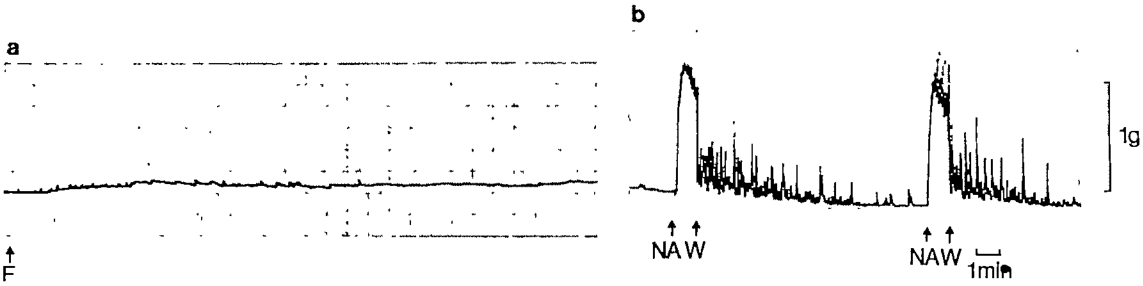

**Figure 4** (a) Tracing from one of six experiments in which 0.2 mM fenfluramine (F) was added to isolated vasa from rats injected 24 h and 48 h previously with reserpine 5 mg/kg. (b) Tissues were subsequently challenged with 5 μM noradrenaline (NA) which was washed out (W) after 1 min exposure. Vertical calibration 1 g, horizontal calibration 1 minute.

by fenfluramine were observed for a short period of time (Figure 4).

## Discussion

Fenfluramine has been shown to reduce noradrenaline concentrations in the brain (Duhault & Verdavainne, 1967; Ziance *et al.*, 1972) and peripherally (Sipes *et al.*, 1971). Mitchell & Mottram (1976) suggested that fenfluramine exerts an effect at adrenergic nerve terminals either by depleting stores of noradrenaline or by inhibiting the amine uptake process, or by a combination of the two.

The present study indicates that, peripherally, fenfluramine exerts its effects by an indirect sympathomimetic action. This conclusion was arrived at by investigating the effect of fenfluramine on isolated vas deferens following, or in the presence of, various pharmacological agents which themselves affect the uptake, storage and release of noradrenaline in sympathetic nerve terminals.

Tyramine is an indirectly acting sympathomimetic amine, which, it has been suggested, releases vesicle-bound noradrenaline (Muscholl, 1966). It has already been shown (Mitchell & Mottram, 1976) that fenfluramine greatly reduces the response to tyramine both in cardiovascular studies and also in isolated tissue. The present study shows that this reduction in response is mutual, in that tyramine pre-treatment of the vas deferens greatly reduces the fenfluramine contractions which are characteristically observed on this tissue. This mutual antagonism by these two drugs may be due either to competition for uptake sites on the nerve terminal membrane, or to depletion of noradrenaline levels from its terminal storage sites. However, it would appear that their respective modes of action on the nerve terminal are not identical, since the response of the vas deferens to each drug, individually, differs in a number of respects. Whilst tyramine-induced contractions in the vas are immediate and short-lived, those produced by fenfluramine require some minutes to appear and are then seen as spiked contractions which require up to 30 min to reach their maximum height.

The effect of neuronal uptake inhibition on fenfluramine responses in the vas deferens was investigated using the potent uptake inhibitor, desipramine (Iversen, 1967). Doses of desipramine (up to 1 μM) which produce an almost complete inhibition of noradrenaline uptake, failed to affect the responses to fenfluramine. The substituent groups on the catechol hydroxyl and nitrogen moieties of fenfluramine indicate that this drug may be unable to utilize the desipramine-sensitive uptake process for noradrenaline. However, at higher concentrations (greater than 0.1 μM) the fenfluramine-induced contractions of the vas were reduced. Westfall (1973), has shown that this concentration of desipramine itself antagonizes the response to noradrenaline on rat vas deferens. This was confirmed in the present study, in that, addition of desipramine (in doses greater than 10 μM) to vasa at the height of their contraction to fenfluramine, produced an immediate reduction in these contractions.

Debrisoquine exerts its neurone blocking effect by inhibiting the release of noradrenaline from the nerve terminals. In the present study, debrisoquine was found to inhibit the fenfluramine-induced contractions of the vas deferens. This inhibition required some time to become established, reflecting the initial, short-acting, noradrenaline releasing property of this group of drugs.

Finally the effect of reserpine treatment on the responses produced by fenfluramine, was investigated. The potent noradrenaline depletion produced by reserpine, resulted in a total blockade of fenfluramine-induced contractions in the vas deferens. The tissues were shown to have maintained their responsiveness to noradrenaline by the exogenous addition of the transmitter which resulted in an immediate contraction of the vasa. Following these additions of noradrenaline and their subsequent washing out from the organ bath, it was noted that fenfluramine-like contractions briefly appeared. It is therefore suggested that these contractions are produced by fenfluramine, which had previously been administered to the tissues and accumulated in the sympathetic nerve terminals, causing a release of exogenous noradrenaline which had been taken up into the nerve terminals during its addition to the bathing fluid.

The results of the present study throw some light on the mode of action by which fenfluramine depletes noradrenaline from tissues both centrally and peripherally. It is suggested that fenfluramine is taken up into adrenergic nerve terminals by a transport process which is not interfered with by the noradrenaline uptake inhibitor, desipramine. Within the nerve terminal, fenfluramine causes the release of the transmitter, noradrenaline, an effect which can be reduced by the acute administration of tyramine, abolished by the chronic administration of reserpine and interfered with by pre-treatment with debrisoquine.

It is concluded that fenfluramine, amongst its other properties, can be classed as an indirectly acting sympathomimetic amine on peripheral adrenergic nerve terminals.

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*(Received September 15, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 621–625

# EVIDENCE FOR A VISCERAL SMOOTH MUSCLE ABNORMALITY IN OKAMOTO SPONTANEOUS HYPERTENSION

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1 In order to discover whether the changes in reactivity are related to the primary cause of hypertension in spontaneously hypertensive rats (SHR) or are just an adaptation induced by the high arterial blood pressure we tested the contractile response of a visceral smooth muscle from such rats.

2 Longitudinal strips of the fundus from 20 week old male and female SHR and Wistar normotensive (NW) rats were used. Dose-response curves to $Ba^{2+}$ in SHR strips were displaced to the right as compared to NW rats. Maximal responses were identical. Male SHR fundus strips contracted much more with $Sr^{2+}$ (SHR: $42 \pm 3\%$ of maximum response to $Ba^{2+}$, $n=10$; NW: $19 \pm 4\%$, $n=10$, $P<0.01$) than NW strips. There was no difference in the response to both $BaCl_2$ and $SrCl_2$ between female SHR and NW fundus strips, and $MnCl_2$ and $LaCl_3$ were relaxant in all cases.

3 Dose-response curves to $Ca^{2+}$ of depolarized SHR and NW fundus strips were obtained and the effect of diazoxide on $Ca^{2+}$ contractions was observed. The contractile action of $Ca^{2+}$ in depolarized preparations was enhanced in both male and female SHR strips. The effect of diazoxide was more marked in SHR strips than in NW fundus strips.

4 SHR fundus smooth muscle shows the same modification of reactivity to $Ba^{2+}$, $Sr^{2+}$, $Ca^{2+}$ and diazoxide that was previously described in arterial smooth muscle. This indicates that the cellular modification responsible for the increase of vascular tonus in SHR is not an adaptive reaction to high blood pressure. The differences between female SHR and male SHR responses are not unexpected, considering the natural evolution of hypertension in Okamoto rats which is milder in the female.

## Introduction

Okamoto & Aoki obtained in 1963 a strain of spontaneously hypertensive rats (SHR) by inbred crossing of male and female Wistar rats which had high arterial blood pressure. This Okamoto strain provides a suitable model for the study of the physiopathology of human essential hypertension. SHR show an increase in vascular resistance that could be due either to increased stimulation of the arterioles by some vasoactive substance (a fact that has not yet been demonstrated) or to a greater reactivity of vascular smooth muscle (Hausler & Finch, 1972). An alternative explanation was given by Folkow, Hallbäch, Lundgren, Sivertsson & Weiss (1972) who suggested that the changes in resistance are due to an increase in the arteriolar wall/lumen ratio. This hypothesis is refuted by the fact that changes in vascular reactivity were also observed in helical strips of SHR arteries. Shibata & Kurahashi (1972) demonstrated that there were qualitative differences in the way SHR aortae responded to some cations. $La^{3+}$ and $Mn^{2+}$, which relax normotensive vascular smooth muscle, contract SHR strips. This finding was confirmed by Bohr (1974) who also showed a decreased sensitivity to $Ba^{2+}$ in SHR carotid arteries. Janis & Triggle (1973) found that diazoxide has a greater inhibitory effect on the response to $Ca^{2+}$ in SHR than in normal aortic strips. These results have been interpreted as indicating that spontaneous hypertension in the Okamoto strain is associated with a defect in $Ca^{2+}$ binding or uptake by some cellular structure. This suggestion was recently confirmed by Moore, Hurwitz, Redman-Davenport & Landon (1975) who showed that Mg-ATP-dependent $Ca^{2+}$ uptake was decreased in SHR aortic microsomes. These authors suggested that increased vascular tonus in SHR may be due to an impaired relaxation mechanism. In all cases, all the observed alterations may result from adaptive changes to an increase in blood pressure, produced by a primary increase in cardiac output (Finkielman, Worcel & Agrest, 1965) induced by various humoral factors (Guyton, Coleman & Granger, 1972). This possibility may have been excluded by Greenberg & Bohr (1975) who observed an increased spontaneous contractility, decreased passive extensibility and a lower threshold for the response to prostaglandins $A_2$ and $B_2$ in SHR portal veins. In order to exclude any haemodynamic influence we have explored the reactivity of SHR extravascular smooth muscle. Experiments performed with SHR stomach fundus strips indicate that spontaneous hypertension in Okamoto rats is associated with a generalized alteration in smooth muscle reactivity that could hardly be explained by an adaptive change to the high blood pressure.

## Methods

We used 20 week old male (blood pressure (BP)= 212 ± 3 mmHg) and female (BP = 155 ± 5 mmHg) Okamoto Aoki Kyoto Wistar rats (SHR). Normotensive Wistar (NW) rats of either sex (male BP= 126 ± 3 mmHg; female BP = 108 ± 2 mmHg) of the same age were used as controls. Blood pressure was measured by a sphygmomanometric plethysmographic method on the tail artery. The animals were killed by a blow on the head, the stomach excised and a longitudinal strip of the fundus was prepared according to the method of Vane (1957). The strips were equilibrated for 2 h in a solution containing (mM): $Na^+$ 137.5, $K^+$ 5.9, $Ca^{2+}$ 2.5, $Mg^{2+}$ 1.2, Tris 15.5, $H_2PO_4$ 1.2, $Cl^-$ 134.1, glucose 11.5 aerated with 100% $O_2$ at 37°C. The strips were attached at one end to a stainless steel holder and at the other to a strain gauge (isometric recording). The initial tension was fixed at 2 grams. The drugs tested were introduced in the 10 ml organ bath in small volumes (0.05 ml).

### *Drugs*

Diazoxide was a gift from the Shering Co, New Jersey, USA. All salts used were reagent grade.

### *Statistical analysis*

The results were compared with a group *t* test and expressed in all cases as the average ± s.e. mean (Schwartz, 1960).

## Results

### *Contractile response to $Ba^{2+}$, $Sr^{3+}$, $Mn^{2+}$ and $La^{3+}$*

All the cations were added in concentrated solution as the chloride salt. In contrast to previous observations on SHR vascular smooth muscles (Bohr, 1974), $Mn^{2+}$ and $La^{3+}$ caused relaxation of fundus strips of both SHR and control animals. Concentration-response curves to $Ba^{2+}$ and $Sr^{2+}$ were obtained cumulatively. $BaCl_2$ was always administered first. Once its maximum response was obtained the strip was washed and left for 60 min in the Krebs solution. $SrCl_2$ was later added cumulatively. Both concentration-response curves were expressed as a percentage of the $BaCl_2$ maximum response. $BaCl_2$ gives a concentration-dependent response with a maximum at 30 mM (Figure 1). $SrCl_2$ has a contractile effect at concentrations higher than 0.3 mM. The actions of both cations were different in male and female SHR. Dose-response curves to $BaCl_2$ on the fundus of male SHR are shifted to the right as compared with controls (Figure 1a); the maximum response is not changed. Responses to $SrCl_2$ are higher in strips from male SHR. On the other hand both $BaCl_2$ or $SrCl_2$ dose-response curves of the fundus of female SHR are identical to control curves (Figure 1b).

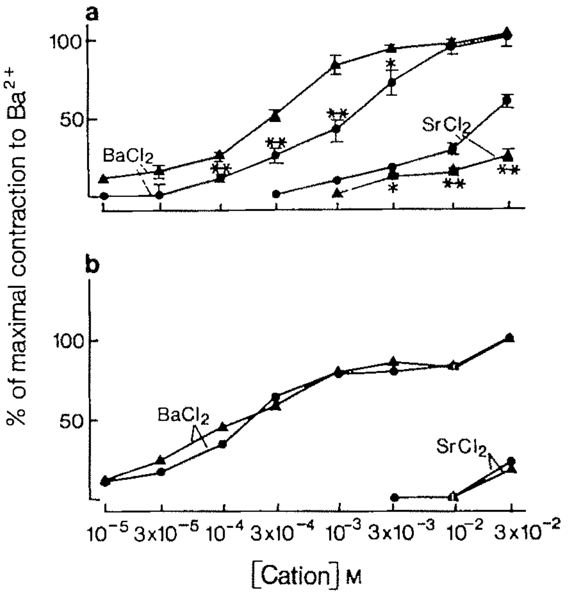

Figure 1 Mean concentration-response curves to $Ba^{2+}$ and $Sr^{2+}$ of rat fundus strips. Cumulative $BaCl_2$ curves were obtained first. $SrCl_2$ was added cumulatively. Responses to $SrCl_2$ are expressed as a percentage of the maximum response to $Ba^{2+}$. (a) Concentration-response curves in strips from male SHR. (b) Concentration-response curves in strips from female SHR. Each point is the mean of 10 experiments. SHR (●); control (▲). The bars represent the s.e. mean. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

### *Contractile response to $CaCl_2$ and relaxant effects of diazoxide*

$Ca^{2+}$ contractions were induced in $K^+$-depolarized strips (Janis & Triggle, 1973). After equilibration with

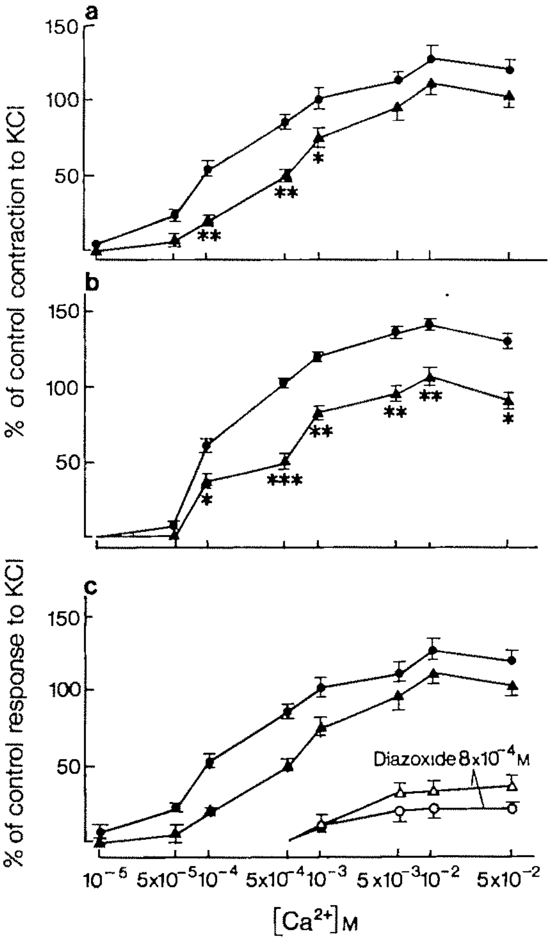

**Figure 2** Cumulative concentration-response curves to $Ca^{2+}$ of depolarized preparations obtained in the presence or in the absence of diazoxide. Contractions were recorded isometrically. A contraction was obtained with 80 mM KCl solution which served as a reference for the following responses. The tissues were then washed with a Krebs solution without $HPO_3$- and $Ca^{2+}$-free. After 100 min, 80 mM KCl was added. When the small response stabilized near the base line, cumulative dose-response curves to $Ca^{2+}$ were obtained in the presence or in the absence of diazoxide. Diazoxide was added 10 min before the first $Ca^{2+}$ dose and maintained throughout the experiment at a fixed concentration. (a) $Ca^{2+}$ concentration-effect curves in male SHR depolarized fundus strips. (b) $Ca^{2+}$ concentration-effect curves in female SHR depolarized fundus strips. (c) Comparison of the response to the cumulative addition of $Ca^{2+}$ to male depolarized fundus strips and male control depolarized strips before and after diazoxide $8 \times 10^{-4}$ M. The points are the mean of 5 to 7 experiments. Dose-response curves with and without diazoxide were performed simultaneously on two strips of the same stomach immersed in separate chambers of the same temperature-controlled bath. SHR (●); control (▲); diazoxide SHR (○), diazoxide control (△).

the normal Krebs solution a contraction was obtained by changing the solution in the organ bath to a 80 mM KCl solution (composition (mM): $Na^+$ 57.2, $K^+$ 80.0, $Ca^{2+}$ 2.5, $Mg^{2+}$ 1.2, Tris 15.5, $H_2PO_4$ 1.2, $Cl^-$ 134.1, glucose 11.5, gassed with 100% $O_2$ at 37°C). This contraction served as a reference for the following responses. The tissues were then washed with a Krebs solution without $HPO_3$ or $Ca^{2+}$ (composition (mM): $Na^+$ 141.2, $K^+$ 5.9, $Mg^{2+}$ 1.2, Tris 15.5, $Cl^-$ 135.3, glucose 11.5, aerated with 100% $O_2$ at 37°C). After 100 min it was replaced by a 80 mM KCl, $Ca^{2+}$ and $HPO_3$-free solution containing (mM): $Na^+$ 66.3, $K^+$ 80.0, $Mg^{2+}$ 1.2, Tris 15.5, $Cl^-$ 135.3, glucose 11.5, aerated with 100% $O_2$ at 37°C. A small contraction was obtained which faded in about 5 minutes. When the tension stabilized near the base line, cumulative dose-response curves to $Ca^{2+}$ were obtained in the presence or in the absence of diazoxide added 10 min before the first $CaCl_2$ dose and maintained throughout the experiment. Diazoxide was dissolved in 0.9% w/v NaCl solution (saline) by a dropwise addition of NaOH solution. The tissues used as controls were treated with the same volume of NaOH-saline solution of the same pH. No effects were detected after the addition of NaOH-saline alone to the tissues. In the fundus of either sex of SHR (Figure 2) there was a significant shift of the $Ca^{2+}$ dose-response curve to the left which indicates that there is an enhancement of $Ca^{2+}$ contractile effects in depolarized SHR preparations. Diazoxide behaved as a non-competitive antagonist of $Ca^{2+}$ in these depolarized strips. In both sexes the antagonist effect was more marked in SHR. Nevertheless strips from female rats were more relaxed by diazoxide than those from males. The drug was tested at concentrations of 1, 2, 4, 6 and $8 \times 10^{-4}$ M. In female strips the antagonist started to be effective at $4 \times 10^{-4}$ M, in male rats at $6 \times 10^{-4}$ M (Table 1).

**Table 1** Reduction of the maximal response to $Ca^{2+}$ of depolarized control and SHR strips produced by diazoxide (% of control response to KCl)

| *Diazoxide* | *Male* | | | *Female* | | |
|---|---|---|---|---|---|---|
| *(M)* | *SHR* | *Control* | P | *SHR* | *Control* | P |
| $4 \times 10^{-4}$ | 8.71 ± 1.3* | 10.8 ± 2.4 | NS | 36.3 ± 2.8 | 16.8 ± 2.1 | 0.01 |
| $6 \times 10^{-4}$ | 12.7 ± 1.5 | 12.0 ± 3.3 | NS | 42.3 ± 3.3 | 19.1 ± 4.0 | 0.001 |
| $8 \times 10^{-4}$ | 101.0 ± 3.8 | 67.5 ± 5.4 | 0.01 | | | |

* Values obtained by averaging the differences of response at $Ca^{2+}$ concentrations of $10^{-3}$, $5 \times 10^{-3}$, $10^{-2}$ and $5 \times 10^{-2}$M, which give maximum contractions. Each value is the average of 5 to 7 experiments.

## Discussion

The present experiments indicate that the changes in reactivity observed in SHR visceral smooth muscle are very similar to the variations in SHR arterial smooth muscle responsiveness reported previously by Holloway, Sitrin & Bohr (1972), Shibata & Kurahashi (1972), Janis & Triggle (1973) and Bohr (1974). However, some differences should be stressed: the decreased response to $BaCl_2$ and the increased effect of $Sr^{2+}$ was observed only in male SHR fundus. Diazoxide was more effective in female tissues. Unfortunately these findings cannot be compared with the results in the literature on vascular smooth muscle, because of the lack of a systematic comparison between male and female specimens. The disparity in $Ba^{2+}$, $Sr^{2+}$ and diazoxide effects is not unexpected considering the fact that the natural history of spontaneous hypertension is rather different in the two sexes. Blood pressure levels in female SHR are lower than in male SHR at all ages. The reason for this feature could be related to the effect of sexual hormones on smooth muscle or to some sex-linked cellular characteristics. We do not think that the haemodynamic changes associated with the lower blood pressure in females is the cause of these findings.

It is difficult to interpret the present results from a physiological point of view, because of the absence of data concerning the mode of action of the cations and diazoxide. Both $Ba^{2+}$ and $Sr^{2+}$ have been shown to compete with $Ca^{2+}$ for binding in vascular smooth muscle (Hudgins & Weiss, 1969), but it is not known whether these cations displace $Ca^{2+}$ from storage sites or troponin. Bohr (1974) observed that in vascular smooth muscle $Ba^{2+}$ contraction is dependent on extracellular $Ca^{2+}$. This suggests that $Ba^{2+}$ contraction is due, at least in part, to a displacement of $Ca^{2+}$ bound to the cell membrane. Possibly there is a decrease of $Ca^{2+}$ binding properties of smooth muscle membranes or other superficial storage sites in SHR rats. Similarly the increased contractile effect of $Ca^{2+}$ in SHR depolarized smooth muscles may be caused by a defect in muscle relaxation. Faulty relaxation may be produced by a decrease of the activity of $Ca^{2+}$ pump located either in the sarcoplasmic reticulum (Moore *et al.,* 1975) or in the cell membrane (Schatzmann, 1973). Alternatively $Ca^{2+}$ efflux from the cell through the $Ca^{2+}$: $Na^+$ exchange mechanism (Brading & Widdicombe, 1976) could be slowed down because of an increase in intracellular $Na^+$ (Jones, 1974). Any of these alterations could be a primary defect of smooth muscle or secondary to modifications in hormonal secretion (Kojima, Kubota, Sato, Yamada, Yamori & Ikamoto, 1975) or to an increase in prostaglandin synthesis as recently suggested by Greenberg (1976).

In conclusion it is suggested that in SHR there is a generalized alteration of the excitation-contraction coupling mechanism in both extravascular and vascular smooth muscles that could hardly be considered as an adaptation to the haemodynamic changes associated with the increase in blood pressure. Hypertension could still play a primary role if it induces humoral disturbances which affect smooth muscle function secondarily, even in extravascular tissues but this hypothesis is far from being demonstrated.

This work was supported by grants from INSERM and DGRST, France. Correspondence should be sent to M.W.

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*(Received September 16, 1976.*
*Revised November 5, 1976.)*

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# TIME COURSE OF LITHIUM-INDUCED ALTERATIONS IN RENAL AND ENDOCRINE FUNCTION IN NORMAL AND BRATTLEBORO RATS WITH HYPOTHALAMIC DIABETES INSIPIDUS

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1 A lithium chloride (1.1 g/kg) supplemented diet was given to Long Evans (LE) and Brattleboro (DI) rats to investigate its actions in the presence (LE) and absence (DI) of vasopressin.

2 During the first 24 h, Li-supplemented LE rats displayed an initial water deficit (drinking less than renal output), increased plasma antidiuretic (ADH) titres and slightly increased plasma renin activities (PRA) and plasma osmolarities. Such changes were qualitatively similar to those seen in rats fed a normal diet, but deprived of water for 24 hours. After 12 days, the Li-supplemented rats had elevated plasma ADH titres, but reduced pituitary oxytocic and antidiuretic activities.

3 The urinary losses of Na, K and Cl exceeded dietary intakes in LE rats on the introduction of the Li-supplement, and the urinary osmolarity fell by 50%. Electrolyte balances were gradually re-established, although drinking and urine production increased in parallel to reach twice the control values by day 12 of the supplement.

4 Aldosterone and corticosterone secretory rates and their peripheral plasma concentrations were unchanged both after 24 h and 28 days of the Li-supplement.

5 Li elicited no water deficit or saluresis in DI rats, and although the polyuria and polydipsia were exacerbated, urinary osmolarity did not change over the 12 day observation period.

6 Li increased Ca excretion in both rat types; after 12 days the PRA of DI but not LE animals was increased.

7 It is concluded that the overall renal actions of Li are tempered by vasopressin rather than adrenocorticosteroids.

## Introduction

The treatment of certain psychiatric disorders with lithium (Li) salts is a well established procedure (Davis & Fann, 1971; Schou & Thomsen, 1975), but the mechanisms involved are not clear. Further there are many physiological processes affected by Li, and undesirable side effects occur including a polydipsic-polyuric syndrome (Singer & Rotenberg, 1973; Forrest, Cohen, Torreti, Himmelhoch & Epstein, 1974). Thus Li influences water and electrolyte metabolism (Forrest *et al.,* 1974) possibly by interfering with vasopressin action (Harris & Jenner, 1972), the renin-angiotensin system (Gutman, Tamir & Benzakein, 1973) and/or with adrenocortical function (Murphy, Goodwin & Bunney, 1969; Hendler, 1975). In addition Li may have direct actions on a variety of renal tubular mechanisms which secondarily influence physiological regulation (Hecht, Galla, Forrest, Kashgarian & Hayslett, 1974; Roscoe, Goldstein, Halperin, Wilson & Stinebaugh, 1976).

The present studies (reported in preliminary form by Balment, Henderson & Chester Jones, 1976b) examine the actions of Li on vasopressin, adrenocortical function and the renin-angiotensin system using intact rats and rats lacking endogenous vasopressin, as a result of an inherited defect in vasopressin synthesis (Sawyer, Valtin & Sokol, 1964). The latter rats have been previously shown to have exacerbated polydipsia and polyuria when given Li (Thomsen & Schou, 1973).

## Methods

### *Animals*

Male and female Long Evans (LE) and Brattleboro (DI) rats, weighing between 160 and 300 g and bred in the Department of Zoology, University of Sheffield, were used.

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### *Experimental protocols*

(i) Groups of 5 rats (male Long Evans and female Brattleboro) were individually adapted to all glass metabolism cages (Jencons Metabowl, Hemel Hempstead) for 10 days, after which daily intakes and urinary excretions of water and electrolytes (Na, K, Ca, Mg, Cl) were measured for 3 consecutive days during which the animals were provided with a normal laboratory chow. A Li-supplemented diet was then substituted and measurements continued daily for a further 12 days. The animals were then anaesthetized with ether, and blood (5–8 ml) was collected from the abdominal aorta into heparinized syringes (in less than 1 minute). Pituitary glands were removed, and like the blood plasma stored at −20° until assay.

(ii) A group of 5 Long Evans male rats were given the Li-supplement for 24 h only and then killed (as in (i) above), to assess more exactly the initial events in the response to lithium. The plasma electrolytes, plasma renin activities, plasma ADH titre and pituitary neurohypophysial peptide stores of these rats were compared with those of normal rats and those of rats killed after 24 h water deprivation, both fed the normal diets.

(iii) Adrenal venous blood was collected from male Long Evans rats 24 h and 28 days after receiving the Li supplement. Appropriate control rats were also studied. Under pentobarbitone anaesthesia (Nembutal, Abbott, 60 mg/kg body wt., i.p.) the left adrenal vein was catheterized (26 g hypodermic needle attached to PE 50 catheter) directly and blood was collected for 10 to 15 minutes. Plasma was stored at −20°C until analysis.

### *Analytical methods and hormone assays*

Pituitary oxytocic activity was assayed on the rat isolated uterus (Holton, 1948), plasma and pituitary antidiuretic activities on the ethanol anaesthetized, water diuretic rat (Dicker, 1953), and plasma renin activity measured indirectly by radio-immunoassay of generated angiotensin I (Stockigt, Collins & Biglieri, 1971). These methods have been previously described for this laboratory (Balment, Henderson & Oliver, 1975; Balment, Chester Jones, Henderson & Oliver, 1976a). Aldosterone and corticosterone in adrenal venous and peripheral arterial blood plasmas were assayed by radioimmunoassays (Mayes, Furayama, Kem & Nugent, 1970; Gross, Ruder, Brown & Lipsett, 1972 respectively). Electrolyte concentrations in plasma and urine were determined by flame emission and absorption spectrophotometry (Pye Unicam SP90A) and their osmolarities on a Fiske Osmometer (Quincy, Mass.).

### *Analysis of data*

The analysis of 24 h water and electrolyte balances has been previously described (Balment *et al.*, 1976a). The balance refers to

$$\frac{\text{urinary loss}}{\text{dietary intake}} \times 100\%$$

so that values in excess of 100% indicate that the animal is excreting more than its dietary intake. In experiment (i) the values observed under the Li regime were compared with the three day control period by paired *t* tests; each animal thus served as its own control. Other comparisons were made using Student's *t* tests, and values are presented as means ± s.e. mean.

### *Diets*

The laboratory diet (B.P., R & M No. 1; B.P. Nutrition (UK) Ltd., Witham, Essex) contained (in mEq/g) 0.119 Na, 0.213 K, 0.277 Ca, 0.145 Mg and 0.182 Cl. The Li-supplemented diet was prepared by incorporating 1.06 g LiCl into each kg of the standard diet as follows: the R & M No. 1 pellets were made into a slurry with distilled water, and 500 ml of a LiCl solution dissolved therein. The mixture was then dried and made into cubes. Control diets were prepared similarly but LiCl was not added. Distilled water to drink and the foods were provided *ad libitum*. The Li-supplement provided on average 0.2 mEq Li per 100 g body wt. daily and gave, after 10 to 12 days, mean plasma Li concentrations of 0.5 mEq/litre.

The following abbreviations are used. ADH = antidiuretic hormone; DI = rats with Diabetes Insipidus; LE = Long Evans rats; Li-supplemented = Lithium supplemented diet; PRA = Plasma renin activity.

## Results

### *Balance studies* (Table 1, Figures 1 and 2)

Plasma composition was largely unaffected in either group of rats on the Li supplement; the only significant change was the mild hypokalaemia of the LE rats ($P < 0.001$) after 12 days. The plasma Li concentrations achieved were similar in the two types of rat.

Food intakes fell transitorily during the first 24 h but normal rates were gradually re-established. Growth continued normally in DI rats, but little weight gain occurred in LE rats during the 12 day observation period. In DI rats an increased drinking rate was detectable during the first 24 h ($P < 0.05$), while no such change occurred in normal rats. However, both types of rat increased their rates of urine production (DI, $P < 0.05$; LE, $P < 0.02$). LE rats were thus in an initial water deficit when Li was added to the diet, and indeed the mean plasma osmolarity increased during this period (Table 1). Figure 1 shows

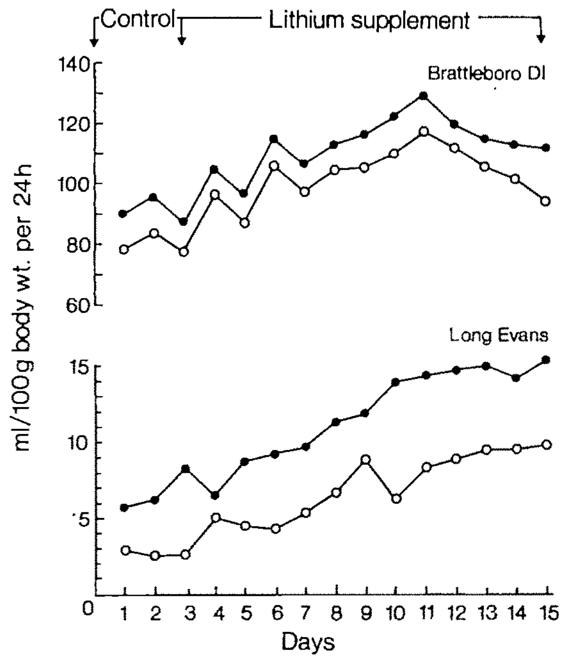

**Figure 1** Effects of a lithium supplemented diet on urine production (O) and drinking (●) rates (ml/100 g body wt. per 24 h) by Long Evans and Brattleboro (DI) rats. A three day control period with normal diet provided, was followed by 12 days with a lithium supplement. Each point is the mean value from 5 rats.

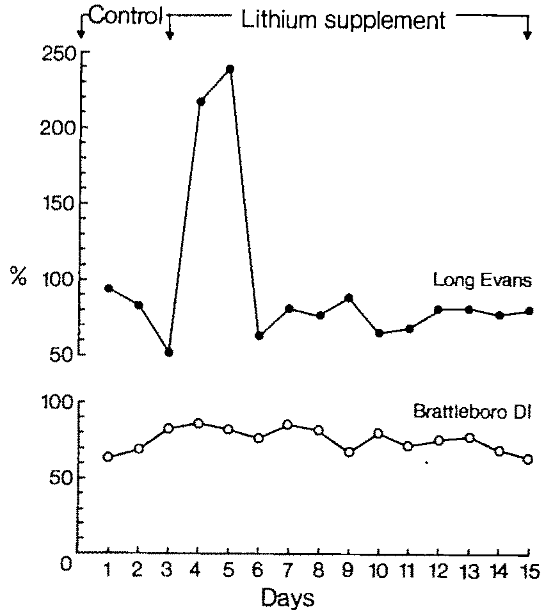

**Figure 2** Effects of a lithium supplemented diet on sodium balance

$$\frac{\text{urinary loss}}{\text{dietary intake}} \times 100\%$$

of Long Evans (●) and Brattleboro (DI) (O) rats. A three day control period with normal diet provided, was followed by 12 days with a lithium supplement. Each point is the mean value from 5 rats.

the sequential changes in water metabolism of the DI and LE rats; except for the somewhat aberrant increase in drinking on control day 3 and the initial 24 h response in LE, parallel changes in intake and output are seen.

In the presence of diabetes insipidus, Li had no effect on urinary osmolarity and solute excretion (Table 1), while in normal rats urine became more dilute and solute excretion increased ($P<0.01$ for both). These changes were associated with little change in the osmotically free water excretion of normal rats, but in DI rats a 25% increase occurred.

Table 1 (part 2) gives electrolyte balances and plasma concentrations of rats 24 h and 12 days after Li supplement. In DI rats the proportion of dietary electrolyte appearing in the urine remained stable throughout the 15 day study (Figure 2; Table 1) except that relatively more K ($P<0.05$, for 3 of the 12 day Li-regime), Ca ($P<0.05$ for 10 days) and Cl ($P<0.05$ for 4 days) appeared in the urine. The time courses of these changes did not follow a clearly decipherable pattern. However, in no instance did the renal loss exceed the dietary intake of individual electrolytes.

In contrast, in LE rats Li affected electrolyte balances. Initially, the Li-supplement produced severe negative balances for Na ($P<0.05$), K (NS) and Cl ($P<0.02$): Figure 2, for Na, typifies the time course. In excess of 200% of the dietary intake of these electrolytes appeared in the urine during this initial period. Thereafter balances gradually returned to more normal, pre-Li levels except for Cl which remained elevated for 6 days ($P<0.05$). Ca excretory patterns were not changed in LE rats, although there was slight retention of Mg ($P<0.05$ for 2 days).

### *Li-responses at 24 h compared with water deprivation* (Table 2)

Plasma Li concentrations in animals given Li for 24 h averaged 0.09 mEq/litre, and during this period LE rats displayed an acute water deficit (see above). This phase of the response was in many respects qualitatively similar to that of rats deprived of water for 24 h, but fed a normal diet. Thus plasma osmolarity ($P<0.001$ dehydrated rats), plasma renin activity ($P<0.001$, dehydrated rats) and plasma antidiuretic titre ($P<0.001$ for both dehydrated and Li rats) were all elevated compared with control animals. Plasma K concentrations were reduced ($P<0.01$,

**Table 1** Twenty-four hour water and electrolyte balances in Long Evans and Brattleboro DI rats on normal and Li supplemented diet for 24 h or 12 days

| *Part 1* | | *Long Evans male* | | | *Brattleboro DI female* | |
|---|---|---|---|---|---|---|
| *Group* | *Control* | *24 h Lithium* | *12 day Lithium* | *Control* | *24 h Lithium* | *12 day Lithium* |
| Food intake (g/100 g body wt.) | 6.0 ± 0.9 | 2.2 ± 1.0 | 6.8 ± 0.7 | 8.9 ± 0.3 | 7.7 ± 0.7 | 9.7 ± 0.7 |
| Water intake (ml/100 g body wt.) | 6.8 ± 0.6 | 6.6 ± 0.9 | 15.3 ± 2.3 | 90.7 ± 5.9 | 104.1 ± 8.4 | 111.0 ± 2.9 |
| Urine output (ml/100 g body wt.) | 2.7 ± 0.5 | 5.0 ± 0.4 | 9.8 ± 2.1 | 80.8 ± 4.3 | 96.1 ± 7.6 | 98.3 ± 3.4 |
| Urinary osmolarity (mOsm/litre) | 1843.0 ± 181.6 | 755.0 ± 154.0 | 935.8 ± 202.0 | 144.6 ± 10.8 | 139.2 ± 9.7 | 140.4 ± 6.9 |
| Free water clearance ($C_{H_2O}$) ml/100 g body wt.) | −12.4 ± 1.6 | −6.9 ± 2.1 | −15.7 ± 3.4 | +42.8 ± 4.8 | +52.9 ± 6.5 | +53.2 ± 2.7 |
| Solute excretion (mOsm/100 g body wt.) | 4.6 ± 0.6 | 3.7 ± 0.6 | 7.8 ± 0.6 | 11.4 ± 0.6 | 13.2 ± 0.4 | 13.8 ± 0.9 |
| *Part 2* | | *Long Evans Male* | | | *Brattleboro DI female* | |
| *Group* | *Control* | *24 h Lithium* | *12 day Lithium* | *Control* | *24 h Lithium* | *12 day Lithium* |
| *Electrolyte balances (%)* | | | | | | |
| Sodium | 76.1 ± 11.8 | 215.8 ± 36.9 | 79.8 ± 3.4 | 71.5 ± 4.8 | 85.4 ± 6.3 | 63.4 ± 4.7 |
| Potassium | 75.3 ± 15.2 | 265.8 ± 88.1 | 80.1 ± 4.6 | 70.7 ± 4.4 | 86.1 ± 4.9 | 70.4 ± 5.0 |
| Calcium | 1.07 ± 0.42 | 2.77 ± 0.89 | 3.61 ± 1.19 | 5.79 ± 0.89 | 9.45 ± 0.73 | 10.13 ± 1.81 |
| Magnesium | 12.33 ± 2.41 | 6.31 ± 4.10 | 9.27 ± 0.95 | 6.99 ± 3.40 | 7.24 ± 2.25 | 8.05 ± 4.23 |
| Chloride | 63.7 ± 5.5 | 185.5 ± 30.3 | 94.8 ± 6.0 | 71.9 ± 3.6 | 85.9 ± 5.0 | 73.5 ± 7.9 |
| *Plasma concentrations* | | | | | | |
| Sodium mEq/litre | 149.1 ± 2.0 | 145.8 ± 0.8* | 146.5 ± 0.6 | 142.6 ± 2.5 | — | 143.8 ± 1.4 |
| Potassium mEq/litre | 4.76 ± 0.1 | 4.36 ± 0.27* | 4.07 ± 0.02 | 3.88 ± 0.15 | — | 3.96 ± 0.15 |
| Lithium mEq/litre | 0.00 | 0.09 ± 0.01* | 0.46 ± 0.01 | 0.00 | — | 0.48 ± 0.02 |
| Osmolarity mOsm/litre | 305.9 ± 0.8 | 313.3 ± 3.9* | 305.5 ± 2.4 | 309.8 ± 1.6 | — | 308.3 ± 0.8 |

5 animals in each group. Values are mean ± s.e. mean.

* Values are also given in Table 2.

**Table 2** Plasma antidiuretic (ADH) and renin (PRA) activities, electrolyte concentrations and osmolarity in normal, 24 h dehydrated and 24 h Li-supplemented male Long Evans rats

| *Group* | *Sodium (mEq/litre)* | *Potassium (mEq/litre)* | *Lithium (mEq/litre)* | *Osmolarity (mOsm/litre)* | *PRA (ng equiv angiotensin I/ml)* | *Plasma ADH (μu/ml)* |
|---|---|---|---|---|---|---|
| Controls<br>$n=5$ | 146.1 ± 0.8 | 4.74 ± 0.19 | 0.00 | 307.4 ± 1.5 | 309.3 ± 13.0 | 133.6 ± 3.8 |
| 24 h Lithium<br>$n=5$ | 145.8 ± 0.8 | 4.36 ± 0.27 | 0.09 ± 0.01 | 313.3 ± 3.9 | 350.0 ± 43.7 | 366.8 ± 12.7 |
| 24 h dehydrated<br>$n=4$ | 150.6 ± 2.4 | 3.92 ± 0.07 | 0.00 | 320.0 ± 4.1 | 466.8 ± 49.0 | 833.7 ± 39.0 |

Values are means ± s.e. mean.

**Table 3** Adrenocortical function in normal and Li-supplemented male Long Evans rats

| *Group* |  | *Corticosterone* |  | *Aldosterone* |  |
|---|---|---|---|---|---|
|  | *Paired adrenal weight* | *Concentration* | *Secretory rate* | *Concentration* | *Secretory rate* |
|  | (mg/100 g body wt.) | (μg/100 ml plasma) | (μg min$^{-1}$ 100 g$^{-1}$ body wt.) | (μg/100 ml plasma) | (ng min$^{-1}$ 100 g$^{-1}$ body wt.) |
| Control | 12.2<br>±0.8<br>(5) | 875.4<br>±98.4<br>(13) | 0.17<br>±0.02<br>(13) | 3.40<br>±0.93<br>(9) | 0.55<br>±0.15<br>(9) |
| 24 h lithium | — | 780.0<br>±74.7<br>(10) | 0.16<br>±0.02<br>(10) | 3.14<br>±0.19<br>(5) | 0.44<br>±0.16<br>(5) |
| 28 day lithium | 13.7<br>±0.5<br>(5) | 791.1<br>±72.1<br>(9) | 0.18<br>±0.02<br>(9) | 1.88<br>±0.56<br>(4) | 0.43<br>±0.12<br>(4) |

Li supplements were provided for 24 h and 28 days before collection of adrenal venous blood. Means ± s.e. mean. Number of animals given in parentheses.

dehydrated rats), and Na concentrations were elevated in the dehydrated group.

### *Adrenocortical function* (Table 3)

Control rat peripheral plasma aldosterone, 38.0 ± 4.4 ng/100 ml, and corticosterone, 12.4 ± 3.1 μg/100 ml, concentrations were similar to those of rats fed Li for 12 days (37.2 ± 2.4 ng aldosterone/100 ml; 9.4 ± 0.6 μg corticosterone/100 ml).

No differences in paired adrenal weights were noted. The concentrations of corticosterone in adrenal venous plasma, the adrenal venous flow and hence its secretory rates were similar in control, 24 h Li and 28 day Li rats (Table 3). Aldosterone was measured in only five 24 h and four 28 day Li rats. In the former group the concentration ranged from 2.6 to 3.6 μg % and from 1.0 to 3.5 μg % in the latter. The secretory rate of aldosterone was not apparently modified by Li (Table 3).

### *Plasma renin activity, pituitary oxytocic and pituitary antidiuretic activities at 12 days*

The PRA, after an initial increase in LE rats, had returned to normal values at 12 days (269.2 ± 28.8 ng angiotensin I equiv/ml). In the DI rats, however, PRA after 12 days was significantly higher in Li rats (356.1 ± 28.4 ng equiv/ml) than in controls (155.6 ± 26.5; $P < 0.001$).

In LE rats, the acute increase (24 h) in antidiuretic titre after Li was sustained to reach 976.4 ± 62.6 μu/ml ($P < 0.001$ compared with untreated controls) after 12 days. There were reduced pituitary antidiuretic and oxytocic activities in Li rats: pituitary antidiuretic activity (in mu/gland) was 706 ± 46.4 in control and 138.8 ± 8.1 in Li rats, while oxytocic activity (mu/gland) was 739.9 ± 18.5 in control and 454.0 ± 37.8 in Li rats ($P < 0.001$ for both).

## Discussion

The polyuric-polydipsic actions of lithium salts are among the important side effects of their application to affective disorders in man (Singer & Rotenberg, 1973; Forrest *et al.*, 1974). In the present studies Li was added to the dried diet, and, apart from the day 1 reduction in food intake, produced a regime akin to the clinical one. This contrasts with other studies in which intraperitoneal or intragastric routes of administration, both stressful procedures, were employed (Baer, Kassir & Fieve, 1970; Martinez-Maldonado, Stavroulaki-Tsapara, Tsaparas, Suki & Eknoyan, 1975) and in which Li was added to the drinking water (Ellman & Gan, 1973). In the latter case, there are obvious difficulties regarding the quantities given, since the induced polydipsia causes the animal to increase its Li load.

The availability of animals precluded the use of a single sex of animals throughout the present study but the Brattleboro DI rats provided a unique opportunity to study the effects of Li in the absence of vasopressin (ADH). In our experience the maintenance of rats in metabolism cages for periods in excess of 2 weeks, after an initial 10 day adaptation period, has no adverse effects on the daily water and electrolyte metabolism (Balment *et al.*, 1976a). The effects of Li were thus assessed from a 3 day control period after adaptation, and a period of Li supplement. Each animal served as its own control and so minimized inter-animal variations.

Many of the sequelae of Li administration have nephrogenic origins. The sensitivity to exogenous vasopressin is diminished (Forrest *et al.*, 1974), while the blockade of endogenous hormone (Harris & Jenner, 1972) may itself provoke the increased water turnover and the reduction in urinary osmolarity of the LE rats. The ion may have direct neural actions which impinge upon the hypothalamo-neurohypophysial system. For example, Li is accumulated in the pituitary gland (Wittrig, Woods & Anthony, 1970) and can deplete the hypothalamo-neurohypophysial system of neurosecretory material (Ellman & Gan, 1973; Hochman & Gutman, 1974). Li may thus interfere with the synthesis, storage and/or release of vasopressin. Indeed Li causes release of vasopressin *in vitro* (Torp-Pedersen & Thorn, 1973). These effects may be associated with, or perhaps be essential components of, the Li actions on CNS sodium fluxes (Baker, 1971). In the present studies significant changes in plasma and pituitary ADH occurred. The plasma values are somewhat high, probably reflecting the method of blood collection (Forsling, Martin, Sturdy & Burton, 1973). Qualitatively, however, LE rats given Li display increased plasma and decreased pituitary ADH compared with control animals. The immediate (within 24 h) increase in plasma antidiuretic titres, and the overall 7-fold increment after 12 days, may be compared with the clinical observation that nephrogenic diabetes insipidus and high plasma vasopressin titres may exist in patients given Li (Padfield, Morton, Lidop & Timbury, 1975).

It is perhaps significant that pituitary oxytocic stores are also depleted by Li and this suggests a general over-stimulation of the hypothalamo-neurohypophysial system. An analogous situation exists in the normal DI rat in which failure to synthesize vasopressin occurs alongside relatively smaller amounts of oxytocin within the pituitary (Balment *et al.*, 1975).

Interference with vasopressin physiology cannot explain the effects of Li on DI rats observed in the present studies and by Thomsen & Schou (1973) and

Hochman & Gutman (1974). Rather, Li may act directly upon thirst mechanisms (Smith & Balagura, 1972), so that the polyuria is secondary (Smith, Balagura & Lubrau, 1970). Thus in the DI rat, Li produces a primary polydipsia, while in LE rats polyuria may be induced perhaps in combination with a central neural action on water intake.

Li may indeed act upon vasopressin-independent aspects of fluid balance, by, for example influencing proximal tubular cation transport (Hecht *et al.*, 1974; Martinez-Maldonado *et al.*, 1975). In the present studies LE rats displayed an initial water deficit resulting from failure to increase drinking in the face of increased renal losses, and elevated plasma vasopressin titres. Concurrently the renal losses of Na, K and Cl exceeded the dietary intakes, features noted in other studies (Tupin, Schlagenhauf & Creson, 1968; Murphy *et al.*, 1969; Baer *et al.*, 1970; Martinez-Maldonado *et al.*, 1975). Neither the fluid nor the electrolyte imbalances occurred in DI rats fed Li. The initial response to Li may thus depend upon vasopressin, although in both LE and DI rats given long term Li, relatively greater percentages of dietary intakes, particularly Cl, appeared in the urine. An additional feature in both LE and DI rats is that the urinary Ca excretion increased, contrasting with the findings of Tupin *et al.* (1968).

Available data are equivocal as to whether Li alters the activity of either the renin-angiotensin system (Gutman *et al.*, 1973; Padfield *et al.*, 1975) or the adrenal cortex (Murphy *et al.*, 1969; Hendler, 1975). Such is perhaps surprising in view of the markedly altered Na and water balances. Adrenocortical function in LE rats was not apparently changed by Li, even after 28 days and the differences in PRA were not statistically significant. The PRA's of DI rats were, however, considerably elevated 12 days after beginning the Li-supplement, but it is premature to speculate on the possible mechanisms involved.

Li thus affects fluid and electrolyte balances in the absence of vasopressin. In the presence of vasopressin the character of the response differs. It is suggested that Li has primary, perhaps direct, actions on neural processes governing thirst, and on renal electrolyte and water transport systems. The inhibition of vasopressin action, both renally and extrarenally must clearly temper the overall Li effect. Vasopressin rather than adrenocorticosteroids modulate the action of Li on the kidney.

R.J.B. is a Science Research Council Post-Doctoral Research Fellow. We are grateful to Dr G. Hargreaves and Miss R. Leonard for the steroid analyses, and Dr J. Ann Brown and Mrs J. Ann Oliver for valuable assistance. Dr H.A. Gross kindly supplied the anti-aldosterone antiserum.

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*(Received September 30, 1976.*
*Revised November 30, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 635–641

# THE PHARMACOLOGY OF ADRENERGIC NEURONAL RESPONSES IN THE CEREBRAL CORTEX: EVIDENCE FOR EXCITATORY $\alpha$- AND INHIBITORY $\beta$-RECEPTORS

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1 The technique of microelectrophoresis was used to compare the actions of a range of adrenoceptor agonists on single cortical neurones in the rat anaesthetized with halothane.

2 Phenylephrine and methoxamine were exclusively excitatory, whereas salbutamol was entirely depressant. Noradrenaline and isoprenaline could evoke both excitatory and depressant responses. Lower doses of isoprenaline usually evoked depressions, whereas higher doses, on the same cell, evoked excitatory responses.

3 The $\alpha$-adrenoceptor blocking agents, phentolamine and phenoxybenzamine, reversibly antagonized excitatory responses to adrenoceptor agonists, without affecting depressant responses to adrenoceptor agonists or excitatory responses to acetylcholine.

4 The $\beta$-adrenoceptor blocking agents, propranolol and sotalol, reversibly antagonized both depressant and excitatory responses to adrenoceptor agonists, without affecting responses to acetylcholine. When the effect of sotalol on excitatory and depressant responses to adrenoceptor agonists was compared on the same cell, the depressant responses could be selectively antagonized, without affecting the excitatory responses.

5 It is concluded that (a) responses of cortical neurones to adrenoceptor agonists are mediated by both $\alpha$- and $\beta$-receptors; (b) these $\alpha$- and $\beta$-receptors give rise to opposite effects: the $\alpha$-receptors being excitatory and the $\beta$-receptors being inhibitory; and (c) responses of many neurones reflect the presence of both types of receptor.

## Introduction

Single cortical neurones are sensitive to noradrenaline (NA) applied by microelectrophoresis: both excitatory and depressant responses have been described (Krnjević & Phillis, 1963; Johnson, Roberts, Sobieszek & Straughan, 1969; Bevan, Bradshaw, Roberts & Szabadi, 1974a). So far, however, these responses have not been characterized in terms of the classical $\alpha$- and $\beta$-adrenoceptors (Ahlquist, 1948).

There are basically three methods available for receptor categorization: comparison of agonists; comparison of antagonists; and desensitization (Schild, 1973). Previous attempts to classify neuronal responses concentrated mainly on the use of antagonists. It has been reported that both $\alpha$- and $\beta$-receptor blocking agents can antagonize excitatory responses to NA whereas depressant responses to NA seem to be much more resistant to these antagonists (Johnson *et al.*, 1969). There are fewer data concerning the effects of different adrenoceptor agonists. Although it has been reported that isoprenaline (IPNA) can evoke both excitatory and depressant responses on cortical neurones (Johnson *et al.*, 1969), the effects of more selective $\beta$-adrenoceptor stimulants (e.g. salbutamol), or the effects of more selective $\alpha$-receptor stimulants (e.g. phenylephrine, methoxamine) have never been tested.

In the experiments presented here we have compared the effects of a range of $\alpha$- and $\beta$-receptor stimulating agents on cortical neurones. We have also re-examined the selectivity of adrenoceptor antagonists. Our results strongly suggest that the excitatory responses of cortical neurones to NA are mediated by $\alpha$- whereas the depressant responses are mediated by $\beta$-receptors.

Some of these results have been presented to the British Pharmacological Society (Bevan, Bradshaw & Szabadi, 1976b).

## Methods

Adult male albino rats (250–300 g) were used. The animals were anaesthetized with halothane (0.8–1.0%). Our methods for the surgical preparation

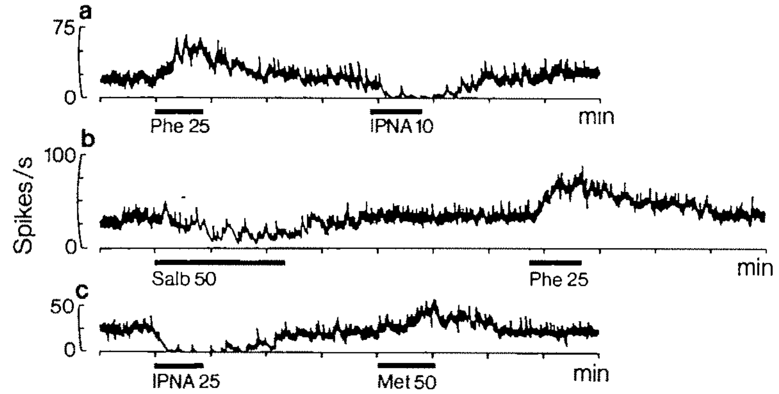

**Figure 1** Examples of the effects of adrenoceptor agonists on cortical neurones. Ratemeter recordings of the firing rates of three cortical neurones in the rat (a, b, c). Ordinate scale: firing rate (spikes/s); abscissa scale: running time (min). Horizontal bars indicate microelectrophoretic drug applications; numbers refer to intensities of ejecting currents (nA). (a) A cell excited by phenylephrine (Phe) and depressed by isoprenaline (IPNA); (b) a cell depressed by salbutamol (Salb) and excited by phenylephrine; (c) a cell depressed by isoprenaline and excited by methoxamine (Met).

of the animals and the manufacture of six-barrelled glass micropipettes for extracellular recording and microelectrophoretic drug application, have been described previously (Bradshaw, Roberts & Szabadi, 1973a; Bradshaw, Szabadi & Roberts, 1973b; Bevan, Bradshaw & Szabadi, 1976a).

Micropipettes having tip diameters of 3–5 μm were used. Two barrels of each micropipette contained 4 M NaCl, one for recording action potentials, the other for use in current balancing. The remaining barrels contained drug solutions. The following drug solutions were used: (–)-noradrenaline bitartrate (0.05 M, pH 3.0–3.5); (–)-phenylephrine hydrochloride (0.05 M, pH 5.0–5.5); (±)-methoxamine hydrochloride (0.05 M, pH 4.5); salbutamol sulphate (0.05 M, pH 5.5); (±)-isoprenaline hydrochloride (0.05 M, pH 5.5); acetylcholine chloride (0.05 M, pH 3.5–4.0); phentolamine mesylate (0.01 M, pH 4.5–5.5); phenoxybenzamine hydrochloride (0.01 M, pH 3.0); propranolol hydrochloride (0.01 M, pH 4.5–5.5); sotalol hydrochloride (0.01 M, pH 4.0–5.0).

Only spontaneously active neurones were studied in these experiments. All the drugs were applied by microelectrophoresis. When a suitable unit was encountered the agonists were applied in a regular cycle. Between successive drug applications retaining currents of –10 nA were passed. Retaining currents of –25 nA were used for the antagonists. Intervals between successive applications of the same agonist were kept constant in order to standardize the effects of the retaining current upon drug release during the ejection period (Bradshaw *et al.*, 1973a,b). The sizes of the neuronal responses to the agonists were expressed as the total number of action potentials produced in response to each drug application ('total spike number', Bradshaw *et al.*, 1973b).

The effects of antagonists were evaluated in the following way. When suitable responses to the agonists had been obtained, the antagonist was applied continuously, either by removal of the retaining current (thus allowing the drug to diffuse out from the micropipette), or by the passage of a weak ejecting current (5–10 nA), and the time course of the developing antagonism was followed. If necessary the intensity of the ejecting current was increased until antagonism was observed. After antagonism of the response to the agonist had been observed, application of the antagonist was continued until a further response to the control agonist(s) could be observed. Then the application of the antagonist was terminated and the time course of recovery was followed. The response to an agonist was regarded as antagonized if there was at least a 50% reduction in the total spike number (Bevan *et al.*, 1974a).

## Results

### *Effects of adrenoceptor agonists*

The proportion of cells responding with excitation (increase in firing rate) or depression (decrease in firing rate) to each adrenoceptor agonist studied are shown in Table 1. It is apparent from the table that phenylephrine and methoxamine were exclusively excitatory, whereas salbutamol was entirely depressant. On the other hand, NA and IPNA could evoke both excitatory and depressant responses. Examples of cells responding both with excitation and depression to different agonists are shown in Figure 1.

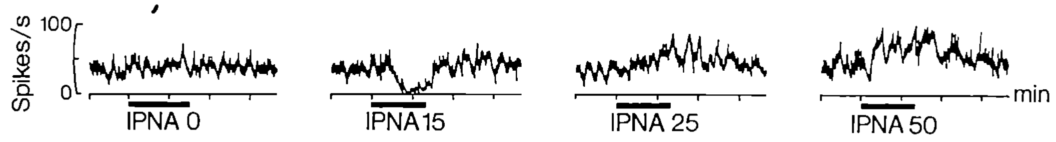

**Figure 2** Responses of a cortical neurone to isoprenaline (IPNA) applied with increasing intensities of ejecting current. Ratemeter recording of firing rate of a single cortical neurone (as in Figure 1). Note the reversal of the response from depression to excitation as the intensity of the ejecting current was increased.

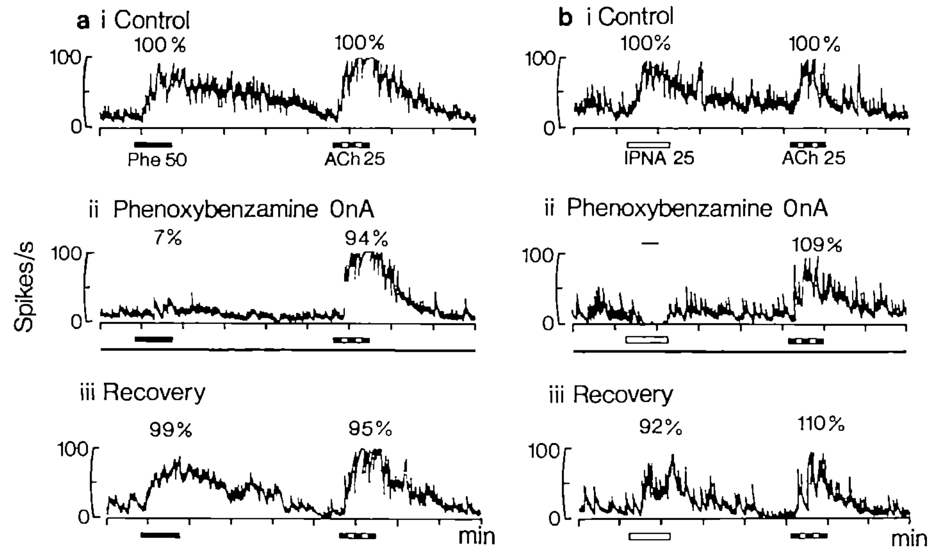

**Figure 3** Effects of phenoxybenzamine on excitatory responses to adrenoceptor agonists and acetylcholine. Ratemeter recordings of the firing rates of two cortical neurones (a) and (b) (as in Figure 1). Figures above the traces indicate total spike numbers (%), taking the sizes of the control responses to each agonist as 100%. (a) Effect of phenoxybenzamine on excitatory responses to phenylephrine (Phe) and acetylcholine (ACh): (i) control responses to the agonists; (ii) responses to the agonists during the continuous application of phenoxybenzamine. Phenoxybenzamine was applied by removal of the retaining current (0 nA), and at the start of trace (ii) had been applied continuously for 5 minutes. The responses to phenylephrine, but not the response to ACh, was antagonized. (iii) Recovery of the response to phenylephrine 15 min after the application of phenoxybenzamine had been terminated. (b) Effect of phenoxybenzamine on excitatory responses to isoprenaline (IPNA) and acetylcholine (ACh): (i) control responses to the agonists; (ii) responses to the agonists during the continuous application of phenoxybenzamine. At the start of trace (ii) phenoxybenzamine (0 nA) had been applied continuously for 12 minutes. The excitatory response to IPNA was antagonized, revealing a depressant response, whereas the response to ACh was not affected. (iii) Recovery of the response to IPNA 10 min after the application of phenoxybenzamine had been terminated.

**Table 1** Percentage of cortical neurones responding either with excitation or depression to adrenoceptor agonists

| | *Percentage of cells responding* | | |
|---|---|---|---|
| | *Excitation* | *Depression* | (n) |
| Phenylephrine | 100 | 0 | 78 |
| Methoxamine | 100 | 0 | 11 |
| Noradrenaline | 66 | 34 | 194 |
| Isoprenaline | 23 | 77 | 138 |
| Salbutamol | 0 | 100 | 16 |

IPNA could evoke both depressant and excitatory responses in a dose-dependent fashion on the same cell: on 14 cells a lower current of IPNA evoked a depression, whereas a higher current evoked an excitation. Examples of this observation are shown in Figure 2. On the basis of this observation, we could predictably evoke depressant responses to IPNA by applying the drug with relatively low ejecting currents (<25 nA) (see the antagonism studies below).

The relative potencies of methoxamine and phenylephrine were compared on 10 cells: in order to obtain approximately equivalent responses to the two

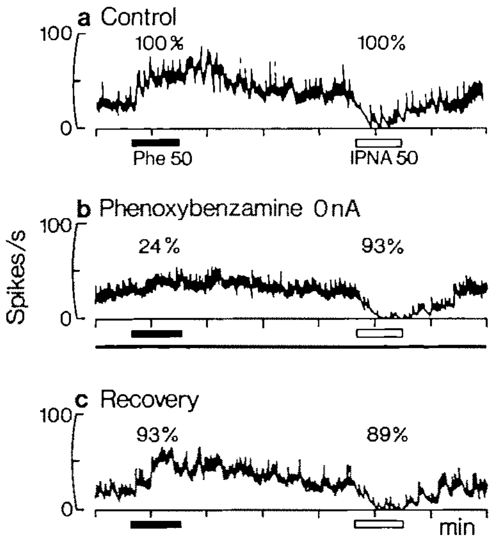

**Figure 4** Effects of phenoxybenzamine on excitatory and depressant responses to adrenoceptor agonists. Ratemeter recording of the firing rate of a single cortical neurone (as in Figure 3): (a) control responses to phenylephrine (Phe) and isoprenaline (IPNA); (b) responses to the agonists during the continuous application of phenoxybenzamine. At the start of trace (b) phenoxybenzamine (0 nA) had been applied continuously for 15 minutes. The excitatory response to phenylephrine, but not the depressant response to IPNA, was antagonized. (c) Recovery of the response to phenylephrine 20 min after the application of phenoxybenzamine had been terminated.

drugs, the current needed to apply methoxamine was at least five times greater than that needed to apply phenylephrine.

### *Effects of adrenoceptor antagonists*

*α-Adrenoceptor blocking agents* The α-adrenoceptor blocking agents, phentolamine and phenoxybenzamine, were effective in reversibly antagonizing excitatory responses to adrenoceptor agonists while excitatory responses to acetylcholine (ACh) were not affected. Phentolamine reversibly and selectively antagonized excitatory responses to NA on 10 cells. Phenoxybenzamine reversibly and selectively antagonized excitatory responses to NA (5 cells), phenylephrine (11 cells), and IPNA (5 cells) (e.g. Figure 3). On one of the cells excited by IPNA, a depressant response was revealed after the abolition of the excitatory response by phenoxybenzamine. On one cell which did not respond to IPNA, a depressant response to IPNA appeared in the presence of phenoxybenzamine; this response disappeared after the application of the antagonist had been terminated.

We have also examined whether the α-adrenoceptor blocking agents can discriminate between excitatory and depressant responses to adrenoceptor agonists on the same cell. In these experiments, excitatory responses were evoked by phenylephrine, depressant responses were evoked by either IPNA or salbutamol, and phenoxybenzamine was used as the antagonist. Antagonism studies were successfully completed on 7 cells; on all these cells the excitatory response was antagonized, whereas the depressant response was not affected. An example of this observation is shown in Figure 4.

*β-Adrenoceptor blocking agents* The β-adrenoceptor blocking agent, sotalol, was effective in reversibly antagonizing depressant responses to IPNA while responses to ACh were not affected (19 cells) (see Figure 5a). On two cells depressed by IPNA an excitation was revealed after the abolition of the depressant responses by sotalol. On one cell which did not respond to IPNA an excitatory response appeared in the presence of sotalol; this response disappeared after the application of sotalol had been terminated. The β-receptor blocking agents, propranolol and sotalol, could also reversibly antagonize excitatory responses to adrenoceptor agonists, without affecting responses to ACh: excitatory responses to NA were antagonized by propranolol on 4 cells, and excitatory responses to IPNA were antagonized by sotalol on 2 cells (see Figure 5b).

We have also examined whether these antagonists can discriminate between depressant and excitatory responses to adrenoceptor agonists on the same cell. In these experiments depressant responses were evoked by IPNA, excitatory responses were evoked by phenylephrine, and sotalol was used as the antagonist. Antagonism studies were successfully completed on 9 cells. On all these cells, the depressant response was antagonized, whereas the excitatory response was not affected (see Figure 6).

## Discussion

The action of adrenoceptor agonists (see Table 1) strongly suggests that the excitatory responses to these drugs are mediated by α-, whereas the depressant responses are mediated by β-receptors. Phenylephrine (Furchgott, 1972; Besse & Furchgott, 1976) and methoxamine (Furchgott, 1970; Innes & Nickerson, 1975) are highly selective α-adrenoceptor agonists, whereas salbutamol is a selective β-receptor stimulant with no affinity for α-adrenoceptors (Brittain, Jack & Ritchie, 1970; Spedding & Weetman, 1972). On the other hand, IPNA and NA can act at both α- and β-receptors (see Furchgott, 1972). It is well documented in the periphery that the dose-response curve to IPNA is a biphasic one: lower

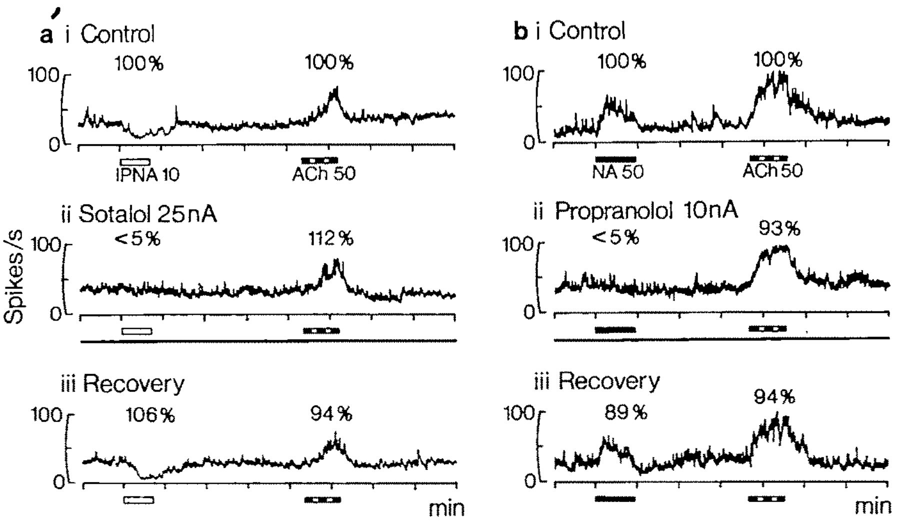

**Figure 5** Effects of $\beta$-adrenoceptor blocking agents on depressant and excitatory responses to adrenoceptor agonists and excitatory responses to acetylcholine (ACh). Ratemeter recordings of firing rates of two cortical neurones, (a) and (b) (as in Figure 3). (a) Effect of sotalol on depressant response to isoprenaline (IPNA) and excitatory response to ACh: (i) control response to agonists; (ii) responses to agonists during the continuous application of sotalol (25 nA). At the start of trace (ii) sotalol and had been applied continuously for 15 minutes. The depressant response to IPNA, but not the excitatory response to ACh, was antagonized. (iii) Recovery of response to IPNA 13 min after the application of sotalol had been terminated. (b) Effect of propranolol on excitatory responses to noradrenaline (NA) and ACh: (i) control responses to agonists; (ii) responses to agonists during the continuous application of propranolol (10 nA). At the start of trace (ii) propranolol had been applied continuously for 9 minutes. The response to NA, but not the response to ACh, was antagonized. (iii) Recovery of the response to NA 5 min after the application of propranolol had been terminated.

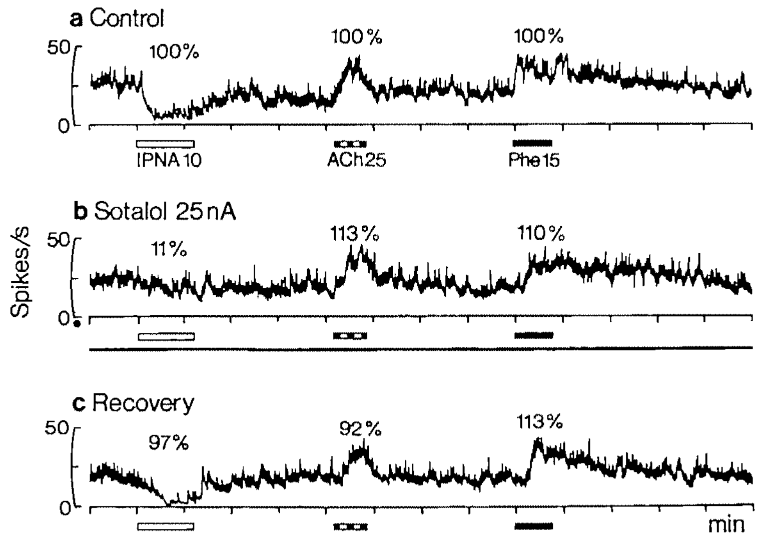

**Figure 6** Effects of sotalol on depressant responses to isoprenaline (IPNA) and excitatory responses to phenylephrine (Phe) and acetylcholine (ACh). Ratemeter recording of the firing rate of a single cortical neurone (as in Figure 3): (a) control responses to the agonists; (b) responses to the agonists during the continuous application of sotalol (25 nA). At the start of trace (b) sotalol had been applied continuously for 35 minutes. The depressant response to IPNA, but not the excitatory responses to either ACh or phenylephrine, was antagonized. (c) Recovery of the response to IPNA 35 min after the application of sotalol had been terminated.

doses of the drug relax smooth muscle preparations due to the stimulation of $\beta$-receptors, whereas higher concentrations have a contractile effect due to the activation of excitatory $\alpha$-receptors (Spedding & Weetman, 1972; Trendelenburg, 1974). A very similar observation was made in our experiments: lower doses of IPNA depressed, whereas somewhat higher doses excited the same cortical neurone (see Figure 2).

Methoxamine appeared to be less potent than phenylephrine in our experiments. This is in agreement with observations in the periphery where methoxamine has a considerably lower potency than phenylephrine (Furchott, 1970; Schümann & Endoh, 1976). However, an apparent difference in potency in our experiments might also reflect physical factors, such as difference between the transport numbers and diffusion coefficients of the two drugs (Szabadi & Bradshaw, 1974).

The results with the adrenoceptor antagonists support our suggestion, based on the action of the agonists, that excitatory responses are mediated by $\alpha$-, and depressant responses are mediated by $\beta$-receptors. In agreement with previous findings (Johnson *et al.*, 1969; Bevan *et al.*, 1974a; Bevan *et al.*, 1976a), we have found that the $\alpha$-adrenoceptor blocking agents could reversibly antagonize excitatory responses to adrenoceptor agonists, while excitatory responses to ACh were not affected. On the other hand, depressant responses to adrenoceptor agonists seemed to be resistant to $\alpha$-adrenoceptor blocking agents (see Figure 4). This observation is in agreement with previous findings (Johnson *et al.*, 1969), and probably reflects the failure of $\alpha$-adrenoceptor blocking agents to interact directly with $\beta$-receptors (Nickerson, 1967). Consequently, we were able to demonstrate that, on the same cell, the antagonism of excitatory responses to phenylephrine occurred when depressant responses to IPNA were not affected (see Figure 4). Occasionally a depressant response could be revealed after the abolition of the excitatory response to IPNA, suggesting an action of IPNA at both types of receptor and also the lower affinity of the antagonist for inhibitory $\beta$-receptors. The selective blockade of excitatory $\alpha$-receptors may explain the observation that a depressant response to IPNA could be revealed by phenoxybenzamine on a cell which previously had not responded to IPNA; the failure of IPNA to evoke a response in the absence of phenoxybenzamine could have been due to a complete 'antagonistic agonism' (Szabadi, 1975) resulting from the activation of the functionally opposite $\alpha$- and $\beta$-receptors.

The $\beta$-adrenoceptor blocking agents effectively abolished depressant responses without affecting excitatory responses to adrenoceptor agonists (see Figure 6). These antagonists occasionally unmasked excitatory responses after the abolition of depressant responses to IPNA, or when IPNA alone was without any effect, indicating the greater sensitivity of the inhibitory $\beta$-receptors. The $\beta$-receptor blocking agents, however, were also capable of antagonizing excitatory responses. This finding is in agreement with previous reports (Johnson *et al.*, 1969; Bevan *et al.*, 1974a; Bevan *et al.*, 1976a). This observation might seem to argue against our hypothesis that the excitatory responses are mediated by $\alpha$-receptors. There is, however, good experimental evidence in the periphery that the conventional $\beta$-receptor blocking agents can also block $\alpha$-receptors at somewhat higher concentrations than are required for selective $\beta$-receptor blockade (Patil, Tye, May, Hetey & Miyagi, 1968; Gulati, Gokhale, Parikh, Udwadia & Krishnamurty, 1969). On the basis of these observations, it might have been expected that higher ejecting currents would be needed to apply the $\beta$-adrenoceptor blocking agents in order to antagonize excitatory responses than to antagonize depressant responses. However, this was not apparent in the present results, and it is unlikely that such a difference could be detected using between-cell comparisons unless a considerably larger number of cells is studied. The differential sensitivities of inhibitory and excitatory receptors to $\beta$-adrenoceptor blocking agents may explain our previous observation that lower doses of sotalol often potentiate excitatory neuronal responses to NA, whereas higher doses have an antagonistic effect (Bevan *et al.*, 1974a; Bevan, Bradshaw & Szabadi, 1974b). The potentiation may reflect the selective blockade of masked inhibitory $\beta$-receptors whereas the antagonism may reflect the blockade of both the $\beta$-receptors and the dominant excitatory $\alpha$-receptors.

Similar to the situation in many peripheral tissues (see Furchgott, 1972), $\alpha$- and $\beta$-receptors mediate opposite effects on cortical neurones, the $\alpha$-receptors being excitatory and the $\beta$-receptors being inhibitory. Moreover, most neurones studied provided evidence for the presence of both $\alpha$- and $\beta$-receptors (e.g. opposite effects of different agonists on the same cell, see Figure 1; opposite effects to different doses of IPNA on the same cell, see Figure 2; reversal of the response by an antagonist, see Figure 3b). Although the experimental evidence presented here does not indicate *where* these receptors are localized, the simplest explanation seems to be that the two functionally opposing populations of $\alpha$- and $\beta$-receptors occur on the membrane of the same cell (Szabadi & Bradshaw, 1974).

This work was supported by the North Western Regional Health Authority and the Mental Health Trust and Research Fund. We are grateful to Mrs E. Markham for technical assistance.

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*(Received October 8, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 643–650

# THE EFFECTS OF LABETALOL (AH 5158) ON ADRENERGIC TRANSMISSION IN THE CAT SPLEEN

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1 The competitive $\alpha$- and $\beta$-adrenoceptor blocking agent labetalol, in concentrations up to $10^{-4}$ M, produced dose-dependent increases in transmitter overflow from the isolated blood perfused spleen of the cat following nerve stimulation at 10 and 30 Hz.

2 At concentrations above $10^{-4}$ M labetalol produced a pronounced decrease in transmitter overflow.

3 Labetalol ($1.5 \times 10^{-4}$ M) increased the recovery of $^3$H label in the venous blood following the close-arterial infusion of [$^3$H]-(–)-noradrenaline indicating that the drug inhibits uptake of the amine.

4 Both labetalol ($3.8 \times 10^{-5}$ M) and piperoxan ($7.4 \times 10^{-6}$ M) produced parallel shifts to the right of the dose-response curves to noradrenaline and oxymetazoline in isolated strips of cat splenic capsule. In this preparation both drugs acted as competitive postsynaptic $\alpha$-adrenoceptor blocking agents.

5 Labetalol ($3.3 \times 10^{-5}$ M) increased the transmitter overflow following stimulation of the splenic nerves with 200 impulses at 10 Hz. The overflow could be further increased by subsequent addition of piperoxan ($7.2 \times 10^{-6}$ M). Piperoxan ($5.7 \times 10^{-6}$ M) alone produced a marked increase in transmitter overflow which could be further increased by subsequent addition of desmethylimipramine (DMI; $3.2 \times 10^{-5}$ M). Cocaine ($1.5 \times 10^{-5}$ M) or DMI ($5.4 \times 10^{-5}$ M) produced a small increase in transmitter overflow which was not further increased by addition of labetalol ($2.8 \times 10^{-5}$ M).

6 Labetalol produced a biphasic effect on the responses of the isolated blood perfused spleen of the cat to nerve stimulation. With low doses (up to $10^{-4}$ M) vascular responses were potentiated and with high doses (greater than $10^{-4}$ M) inhibited. The potentiation was related to uptake blockade and the inhibition to decreased transmitter overflow and postsynaptic $\alpha$-adrenoceptor blockade.

7 Labetalol appears to act as a postsynaptic $\alpha$-adrenoceptor antagonist in the isolated blood perfused spleen of the cat with little effect on presynaptic $\alpha$-adrenoceptors. The moderate elevation of transmitter overflow by the drug is related to the inhibitory effect of the drug on neuronal uptake rather than on presynaptic $\alpha$-adrenoceptors.

## Introduction

Release of noradrenaline from sympathetic nerve endings in response to nerve stimulation is controlled by a local feedback mechanism. This feedback is believed to be mediated by $\alpha$-adrenoceptors located on the presynaptic nerve terminals (Kirpekar & Puig, 1971; Starke, 1972; Enero, Langer, Rothlin & Stefano, 1972; Langar, 1974).

Pre- and post-synaptic $\alpha$-adrenoceptors appear to differ in their susceptibility to drugs. In the rabbit pulmonary artery the relative potencies of $\alpha$-adrenoceptor agonists at the two sites were found to vary; methoxamine and phenylephrine were found to act preferentially on postsynaptic $\alpha$-adrenoceptors; noradrenaline, adrenaline and naphazoline have similar pre- and post-synaptic agonist potencies; and oxymetazoline, $\alpha$-methyl noradrenaline, tramazoline and clonidine acted preferentially at presynaptic $\alpha$-adrenoceptors (Starke, Montel, Gayk & Merker, 1974; Starke, Endo & Taube, 1975). In the guinea-pig vas deferens, lysergic acid diethylamide appears to act as a selective presynaptic $\alpha$-adrenoceptor agonist (Hughes, 1973). Among $\alpha$-adrenoceptor antagonists similar preferential blockade of one of the two types of $\alpha$-adrenoceptor can be obtained. Phenoxybenzamine is a more effective blocker of postsynaptic $\alpha$-adrenoceptors in rat portal vein and cat spleen (Haggendal, Johansson, Jonason & Ljung, 1972; Dubocovich & Langer, 1974). In the rabbit pulmonary artery (Borowski, Ehrl & Starke, 1976) the antagonists ranged in their effects from the post-synaptic $\alpha$-adrenoceptor blocker clozapine through azapetine, mianserin, phentolamine, dihydroergotamine, piperoxan and tolazoline to a predominantly presynaptic $\alpha$-adrenoceptor blocker, yohimbine.

Labetalol is an adrenoceptor blocking drug which produces a competitive blockade of both $\alpha$- and $\beta$-adrenoceptors (Farmer, Kennedy, Levy & Marshall, 1972; Kennedy & Levy, 1975). The following experiments provide evidence to show that labetalol is an antagonist at postsynaptic $\alpha$-adrenoceptors with little effect on presynaptic $\alpha$-adrenoceptors in the isolated blood perfused spleen of the cat. A preliminary account of these results has been published (Blakeley & Summers, 1976).

## Methods

### *Overflow of endogenous transmitter*

Cat spleens were perfused with blood *in vitro* (Blakeley, Brown, Dearnaley & Woods, 1969; Blakeley, Powis & Summers, 1973). The splenic nerves were stimulated with shielded bipolar platinum electrodes. Supramaximal stimuli of 20 V and 0.5 ms duration were used throughout.

The plan of the experiments in which the effects of labetalol on transmitter overflow were investigated was as follows. Transmitter overflow was stabilized by giving several conditioning trains of stimuli (Bacq, Blakeley & Summers, 1976). Transmitter overflow was then measured following two trains of 200 stimuli at 10 Hz and two similar trains at 30 Hz. On some occasions only 30 Hz was used. Labetalol was then added to the blood in 0.9% w/v NaCl solution (saline) and allowed 20 min to act. Transmitter overflow was then measured following two further periods of stimulation at either 10 or 30 Hz. In any one experiment up to 4 cumulative doses of labetalol were used and the effect on transmitter overflow measured after each dose increment.

Overflowing transmitter in the venous blood was collected for 1 min following stimulation at 30 Hz and for 80 s following stimulation at 10 Hz. The blood collected was chilled, spun and the transmitter in the plasma assayed against (–)-noradrenaline on the blood pressure of the pithed rat (Shipley & Tilden, 1947).

### *Uptake of [$^3$H]-(–)-noradrenaline*

Uptake of [$^3$H]-(–)-noradrenaline was measured from infusions given close arterially, at a rate of 360 ng/min, to the spleen (perfusion rate $6.5 \pm 0.6$ ml/min, $n=4$). Uptake was taken as the difference between the amount of label given and the amount recovered in the venous blood. This method of measuring uptake entails the acceptance of some errors. Any infusate which is lost through minor bleeding from the spleen, trapped in areas of poor perfusion, or trapped in the red cell layer of the centrifuged samples will tend to elevate uptake when measured by this method. In order to check that this error was not large in any experiment, Evans Blue was added to the infusate to act as an intravascular marker. In the present series of experiments the overall recovery of Evans Blue was $93.7 \pm 4.5\%$ ($n=4$). The results have not been corrected for this recovery.

### *Isolated strips of cat splenic capsule*

Cats (2.4–6 kg) were anaesthetized with 1.5% halothane in $N_2O/O_2$ (2:1 v/v); spleens were removed and washed in Krebs-Bicarbonate solution of the following composition (mM): $NaHCO_3$ 25, NaCl 120, KCl 4.5, $NaH_2PO_4$ 0.19, $Na_2HPO_4$ 1.83, $CaCl_2$ 1.25, $MgSO_4$ 1.00 and glucose 11.1. A 0.5 mm thick layer of the outer capsule of the spleen was sliced off with a modified Stadie–Riggs tissue slicer. The capsular strip was washed with warm Krebs solution and sliced along the long axis of the spleen into pieces measuring approximately 35 × 4 mm. Pairs of strips were set up in a 25 ml organ bath containing Krebs solution bubbled with 95% $O_2$ and 5% $CO_2$ and maintained at 38°C. Contractions of the strips were recorded isotonically under 1 g tension using d.c./d.c. linear variable differential transformer type transducers (Sangamo Weston Controls Ltd. : type ND1 ± 5 mm stroke) the outputs of which were displayed on a u.v. recorder (SE 3006). Cumulative dose-response curves were obtained for both noradrenaline and oxymetazoline. It was possible to wash out the noradrenaline after obtaining one dose-response curve and repeat the procedure and obtain another dose-response curve which did not differ significantly from the first. Therefore, in the experiments with noradrenaline as the agonist, the $\alpha$-adrenoceptor antagonist was added between exposures to noradrenaline in the same tissue. However, with oxymetazoline, it was not possible to wash out the drug, so that a comparison between the effects of the $\alpha$-adrenoceptor antagonists using this agonist was performed on preparations taken from adjacent areas of the same spleen.

### *Drugs*

[7-$^3$H]-(–)-noradrenaline (9.8 mCi/μM); Radiochemical Centre, Amersham) was diluted with (–)-noradrenaline (Sigma) to give a specific activity of 248 μCi/μM; the following drugs were also used: oxymetazoline hydrochloride, Evans Blue (E. Merck, Darmstadt), piperoxan (2-piperidinomethyl-1,4-benzodioxane hydrochloride; Rhone-Poulenc, Paris), cocaine hydrochloride (E. Coburn Ltd.), labetalol (5-(1-hydroxy-2[(1-methyl-3 phenylpropyl)amino] ethyl) salicylamide; Dr G.P. Levy, Allen and Hanbury's Research Ltd.), desmethylimipramine hydrochloride (Pertofran: Geigy Ltd.), heparin (mucus; Boots Ltd.) and prostaglandin $E_1$ (Dr John E. Pike, Upjohn, Kalamazoo, U.S.A.). All doses of amines are expressed as base.

Unless otherwise defined results are presented as means ± s.e. mean. Significance was assessed by Student's *t* test.

## Results

### *Stabilization of transmitter overflow*

The overflow of transmitter following nerve stimulation falls to a plateau after two to three periods of stimulation (Blakeley *et al*, 1969). Series of conditioning stimuli were, therefore, given at the start of each experiment (Bacq *et al.*, 1976). The conditioning periods of stimulation were followed by several control periods of stimulation during which transmitter was collected and assayed. In experiments where the effect of labetalol on overflow following stimulation at 10 Hz was examined, two control periods of stimulation at 30 Hz and two at 10 Hz were given and the transmitter overflows measured. In experiments where the effect of the drug on overflow at 30 Hz was studied only the two periods of stimulation at 30 Hz were given. Table 1 shows that the conditioning periods of stimulation effectively stabilized transmitter overflow following stimulation at both 10 and 30 Hz.

### *The effect of labetalol on transmitter overflow following nerve stimulation*

Labetalol in concentrations up to $10^{-4}$ M produced a dose-dependent elevation of transmitter overflow at both frequencies of stimulation as shown in Figure 1.

In these experiments transmitter overflow has been expressed as the ratio of the overflow in the presence of the drug compared with the mean overflow obtained after the control periods of stimulation at 30 Hz in the same experiment ($0/\bar{0}_{30}$). This form of expression of transmitter overflow has been used because although the overflow in any one experiment is reproducible, the variation in overflow obtained in different spleens is large (see Table 1 and Blakeley *et al.*, 1969). The elevation of overflow produced by labetalol at 10 Hz was greater than that obtained at 30 Hz so in this respect it resembles other α-adrenoceptor antagonists such as dibenamine and phenoxybenzamine (Brown & Gillespie, 1957).

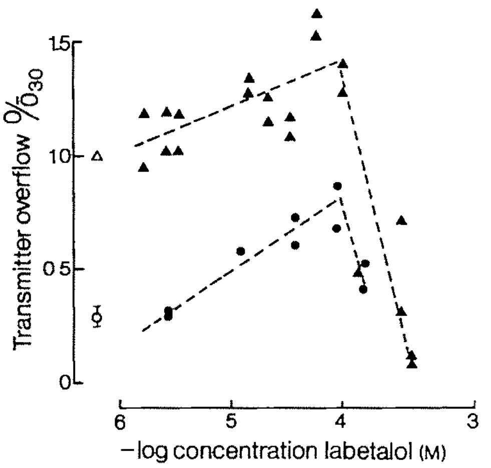

**Figure 1** The effect of labetolol on the overflow of transmitter following nerve stimulation in the isolated blood perfused spleen of the cat. Open symbols represent control overflows of transmitter ($O/\bar{O}_{30}$) at 10 Hz (○) and 30 Hz (△) and solid symbols overflows of transmitter in the presence of labetalol following 200 stimuli at 10 Hz (●) and 30 Hz (▲).

At concentrations above $10^{-4}$ M labetalol produced a pronounced depression of transmitter overflow. We have attributed this effect to the local anaesthetic properties of the drug in high concentrations (Farmer *et al.*, 1972).

### *The effect of labetalol on uptake of [$^3$H]-(−)-noradrenaline infused into the cat spleen*

[$^3$H]-(−)-noradrenaline was infused close arterially into cat spleens at a rate of 360 ng/minute. In the blood perfused cat spleen a steady state condition is reached

**Table 1** Effect of conditioning stimulation (3 x 200 stimuli at 10 Hz followed by 2 x 200 stimuli at 30 Hz) on transmitter overflow during control periods of stimulation in the isolated blood perfused spleen of the cat.

| | *Overflow of transmitter during control periods of stimulation* | | | | | |
|---|---|---|---|---|---|---|
| | *pg/stimulus* | n | P | *$O/\bar{O}_{30}$* | n | P |
| 1st stimulation (200 at 30 Hz) | 1195 ± 108 | 25 | >0.7 | 1.02 ± 0.013 | 25 | <0.05 |
| 2nd stimulation (200 at 30 Hz) | 1140 ± 95 | 25 | | 0.98 ± 0.013 | 25 | |
| 3rd stimulation (200 at 10 Hz) | 374 ± 51 | 17 | >0.8 | 0.33 ± 0.036 | 17 | >0.8 |
| 4th stimulation (200 at 10 Hz) | 364 ± 42 | 17 | | 0.32 ± 0.031 | 17 | |

Overflow expressed as pg/stimulus or as a fraction of the average overflow from the two control periods of stimulation at 30 Hz ($O/\bar{O}_{30}$).

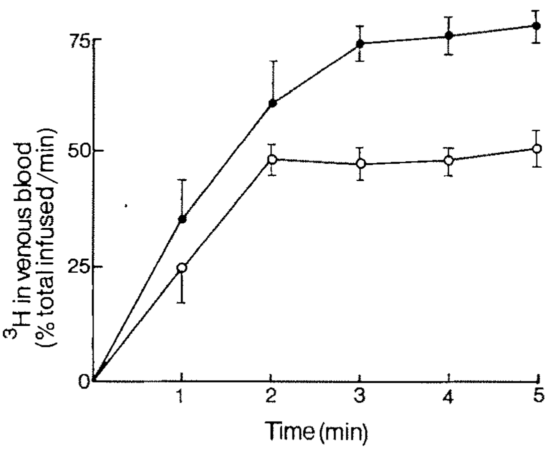

**Figure 2** The effect of labetalol on recovery of $^3$H in the venous blood following close arterial infusion of [$^3$H]-(−)-noradrenaline (360 ng/min) to the isolated blood perfused spleen of the cat. Open symbols (○) represent the recovery of $^3$H in the absence of drug and the filled symbols (●) the recovery in the presence of labetalol ($1.5 \times 10^{-4}$ M). $n=4$ in each case.

within 2 to 3 min of the start of the infusion (Blakeley, Powis & Summers, 1974). In the absence of drugs the recovery of label infused into the spleen between the 3rd and 5th minutes of infusion was $48.9 \pm 1.9\%$ ($n=12$) as shown by the open symbols in Figure 2. In the presence of labetalol ($1.5 \times 10^{-4}$ M) the recovery of the label in the venous blood is increased to $76.0 \pm 2.0\%$ of that administered ($n=12$; $P<0.001$). The increase in recovery of label in the venous blood indicates that less has been taken up by the spleen and hence it is likely that labetalol is an uptake inhibitor.

### *The effect of labetalol and piperoxan on the responses of isolated strips of splenic capsule to noradrenaline and oxymetazoline*

Labetalol is known to antagonize competitively α-adrenoceptor mediated responses in the guinea-pig mesenteric vein, the rat vas deferens (Farmer *et al.*, 1972) and of the blood pressure in the anaesthetized dog (Kennedy & Levy, 1975). Piperoxan is a classical competitive α-adrenoceptor antagonist (Bacq & Fredericq, 1935; Ariens, 1967) with no inhibitory effects on metabolism or uptake of noradrenaline (Blakeley & Summers, 1975). Both labetalol and piperoxan produced parallel shifts to the right of the dose-response curve to noradrenaline in cat spleen strips as shown in Figure 3a. The doses used were close to those which produced the maximal elevating effect on transmitter overflow.

The shift to the right of the dose-response curve to noradrenaline at the level of the $ED_{50}$ with labetalol ($3.8 \times 10^{-5}$ M) was 5.6-fold whereas with piperoxan ($7.4 \times 10^{-6}$ M) it was 13.0-fold. Since, in addition to its α-adrenoceptor blocking properties, labetalol is an inhibitor of noradrenaline uptake and inhibitors of uptake shift the dose-response curve to noradrenaline in spleen strips to the left (Davidson & Innes, 1970; Granata & Langer, 1973; Guimaraes & Brandao, 1973), it is likely that experiments with noradrenaline do not give a true reflection of the α-adrenoceptor blocking properties of the drug. Therefore, in addition to the experiments with noradrenaline as an agonist, experiments were performed using another α-

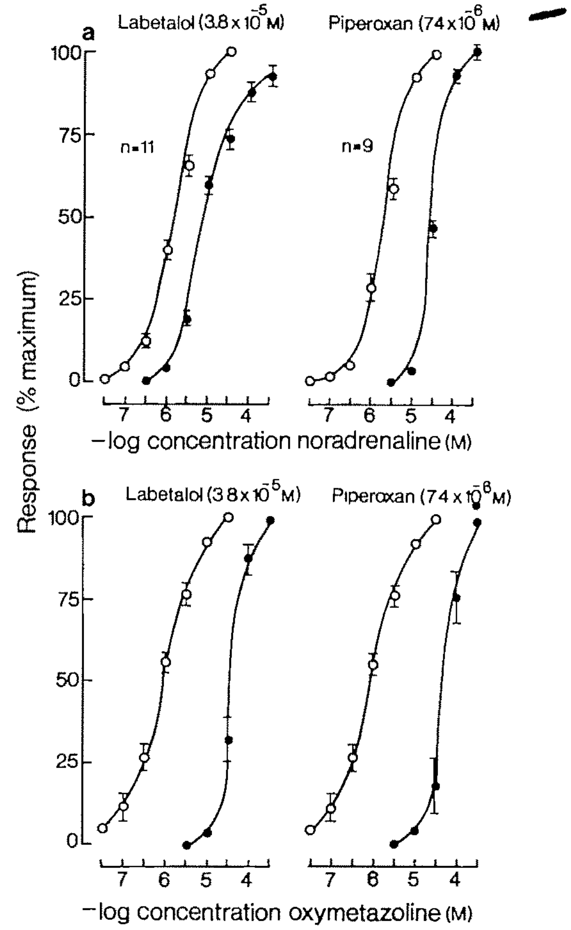

**Figure 3** The effect of labetalol and piperoxan on responses of isolated strips of cat splenic capsule (a) to noradrenaline and (b) oxymetazoline. In (a) dose-response curves to noradrenaline were obtained in the same strips before (○) and after (●) exposure to labetalol ($3.8 \times 10^{-5}$ M) or piperoxan ($7.4 \times 10^{-6}$ M) with a 40 min wash between. In (b) the dose-response curves to oxymetazoline were obtained in separate spleen strips in the presence of (●) or in the absence of (○) labetalol or piperoxan, since oxymetazoline does not easily wash out of this preparation.

adrenoceptor agonist, oxymetazoline (Mujic & Van Rossum, 1965) which is not a substrate for uptake$_1$ (Birmingham, Paterson & Wojcicki, 1970). In these experiments, shown in Figure 3b, both labetalol ($3.8 \times 10^{-5}$ M) and piperoxan ($7.4 \times 10^{-6}$ M) produced similar parallel shifts to the right of the dose-response curve, and labetalol was 5.1 times less potent than piperoxan. The experiments provide evidence to show that both labetalol and piperoxan are competitive postsynaptic α-adrenoceptor antagonists in isolated strips of cat splenic capsule.

### *Investigation of the mechanism of the effect of labetalol on transmitter overflow*

The elevation of overflow produced by labetalol is likely to involve inhibition of (−)-noradrenaline uptake or blockade of presynaptic α-adrenoceptors or both. The effects of labetalol on transmitter overflow were therefore compared with those of the known uptake$_1$ blockers DMI and cocaine, and with a presynaptic α-adrenoceptor antagonist without uptake blocking properties, piperoxan (Blakeley & Summers, 1975). The additive effects of labetalol with these was also investigated. The results of these experiments are presented in Table 2. Labetalol and piperoxan individually elevated transmitter overflow and their effects were additive. After uptake$_1$ blockade, labetalol produced no further increase in overflow (changes greater than 25% should have been detected in these experiments whereas a presynaptic α-adrenoceptor blocker might have been expected to produce an increase greater than 300%). The doses of piperoxan used were both supramaximal for their elevating effect on transmitter overflow (Blakeley & Summers, 1975). The slope of the dose-response curve for labetalol (see Figure 1) is not steep and a similar effect would have been expected with both doses used.

### *Effect of labetalol on responses of the spleen to nerve stimulation*

Following stimulation of the splenic nerves with 200 supramaximal stimuli at 10 Hz the spleen responds with increases in perfusion pressure (vascular responses) and with contractions of the splenic capsular smooth muscle (seen as increases in venous blood flow) as shown in Figure 4a.

As with the effect on transmitter overflow, labetalol produced a biphasic effect on vascular and capsular responses of the spleen to nerve stimulation. At low doses, responses were potentiated and at high doses, inhibited. The potentiation observed with labetalol (Figure 4b) is not as pronounced as that observed with cocaine in this preparation (Cripps & Dearnley, 1972) and probably represents the resultant of the inhibition of uptake and postsynaptic α-adrenoceptor blockade. The inhibition of responses (Figure 4c) with higher doses is probably due to the inhibitory effect of the drug on release of transmitter and the postsynaptic α-adrenoceptor blocking effect.

## Discussion

The isolated blood perfused spleen of the cat has been

**Table 2** The effect of drugs on the overflow of transmitter in the isolated blood perfused spleen of the cat following nerve stimulation with 200 stimuli at 10 Hz.

| | *Drug concentration (M)* | *Transmitter overflow* $(0/\bar{0}_{30})$ | n | P |
|---|---|---|---|---|
| Control | – | 0.32 ± 0.02 | 21 | <0.001 |
| Labetalol | $3.3 \times 10^{-5}$ | 0.75 ± 0.09 | 8 | <0.001 |
| Labetalol<br>+ Piperoxan | $3.3 \times 10^{-5}$<br>$7.2 \times 10^{-6}$ | 1.44 ± 0.12 | 8 | <0.05 |
| Piperoxan | $5.7 \times 10^{-6}$ M | 1.11 ± 0.08 | 14 | <0.05 |
| Piperoxan<br>+ DMI | $5.7 \times 10^{-6}$ M<br>$3.2 \times 10^{-5}$ M | 1.45 ± 0.17 | 4 | |
| DMI<br>or cocaine | $5.4 \times 10^{-5}$<br>$1.5 \times 10^{-6}$ | 0.48 ± 0.04 | 8 | >0.3 |
| DMI<br>or cocaine<br>+ labetalol | $5.4 \times 10^{-5}$<br>$1.5 \times 10^{-5}$<br>$2.8 \times 10^{-5}$ | 0.42 ± 0.04 | 8 | |

Overflow is expressed as a fraction of the mean overflow obtained after two control periods of stimulation at 30 Hz $(0/\bar{0}_{30})$.

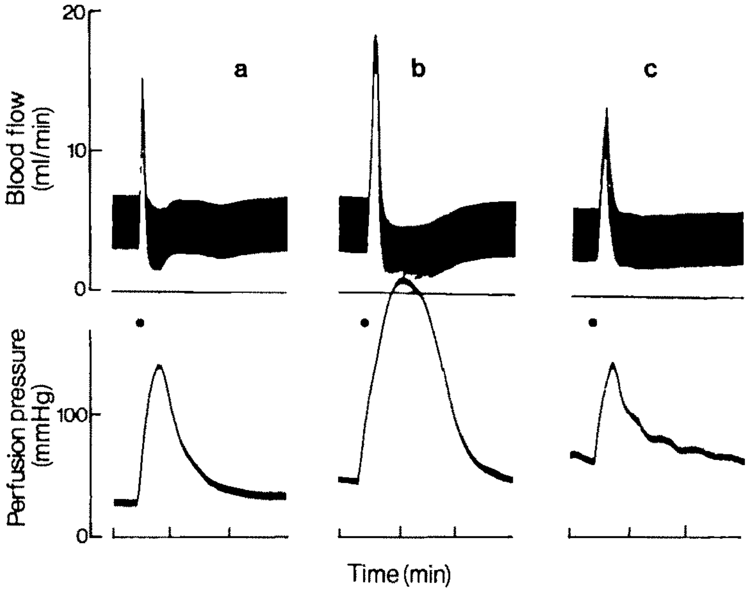

**Figure 4** The effects of labetalol on the change in vascular resistance and venous blood flow in the isolated blood perfused spleen of the cat following nerve stimulation with 200 stimuli at 10 Hz. (●). (a) Control; (b) 20 min after labetalol ($2.1 \times 10^{-5}$ M); (c) 20 min after labetalol ($3.4 \times 10^{-4}$ M). Time marks, 1 minute.

used to examine the pharmacology of labetalol, a drug which has the unusual property of being a competitive blocker of both $\alpha$- and $\beta$-adrenoceptors (Farmer *et al.*, 1972). Many $\alpha$-adrenoceptor antagonists such as dibenamine (Brown & Gillespie, 1957), hydergine (Blakeley, Brown & Ferry, 1963; Cripps & Dearnaley, 1972), phentolamine (Kirpekar & Puig, 1971; Cubeddu, Barnes, Langer & Weiner, 1974) and piperoxan (Blakeley & Summers, 1976), are known to increase transmitter overflow following stimulation of the splenic nerves. There is now abundant evidence that these drugs elevate overflow by blockade of an inhibitory feedback loop acting via $\alpha$-adrenoceptors located on the presynaptic nerve terminals (Kirpekar & Puig, 1971; Starke, 1972; Enero *et al.*, 1972; Langer, 1974).

The present experiments show that labetalol increases transmitter overflow on nerve stimulation. The mechanism of this elevation does not involve inhibition of presynaptic $\alpha$-receptors. The evidence for this conclusion was that the preferential presynaptic $\alpha$-adrenoceptor blocker, piperoxan (Borowski, *et al.*, 1976) given after labetalol produces a further increase in overflow and the fact that labetalol given after uptake$_1$ blockade fails to produce any marked increase in overflow. The overflow obtained in the presence of both labetalol and cocaine was less than that obtained with labetalol alone. The reason for this may lie in the patterns of overflow obtained after treatment of the spleen with these drugs. Labetalol alone produced no change in the pattern of overflow of transmitter. Cocaine on the other hand considerably prolongs the overflow of transmitter (Cripps & Dearnaley, 1972; Cubeddu *et al.*, 1974) so that collection of venous blood for 80s would result in collection of only a fraction of the total transmitter overflowing.

Those results obtained with cocaine and DMI also suggest that the mechanism whereby labetalol increases overflow is blockade of uptake$_1$. There is now evidence which supports this conclusion. In the present experiments in the spleen, labetalol increased the recovery of $^3$H in the venous blood following infusion of [$^3$H]-(−)-noradrenaline: the drug has been shown to block a cocaine-sensitive uptake process in the anaesthetized dog (Farmer *et al.*, 1972) and it inhibits production of the neuronally produced metabolite 3 : 4 dihydroxyphenyl-ethylene glycol in the cat spleen (Summers & Tillman, 1976). The experiments in spleen strips also support this conclusion. Labetalol was a less potent antagonist of noradrenaline than piperoxan, both drugs being given in concentrations that were optimum for elevating overflow. However, when the agonist used was oxymetazoline, an $\alpha$-adrenoceptor agonist (Mujic & Van Rossum, 1965) which is not a substrate for uptake$_1$ (Birmingham *et al.*, 1970), both drugs were similar in their effectiveness as $\alpha$-receptor blockers. It is likely that when noradrenaline is the agonist the uptake blocking properties of labetalol prevent its true $\alpha$-adrenoceptor blocking properties from being seen.

The experiments in spleen strips confirm that labetalol is a postsynaptic $\alpha$-adrenoceptor blocker in the cat spleen as in guinea-pig mesenteric vein, rat vas deferens (Farmer *et al.*, 1972) and on dog blood pressure (Kennedy & Levy, 1976). This would indicate that in the cat spleen labetalol is a preferential antagonist of postsynaptic $\alpha$-receptors.

However, there are other explanations which must be considered. The first concerns the presence of a $\beta$-adrenoceptor mediated positive feedback mechanism at adrenergic nerve endings (Adler-Graschinsky & Langer, 1975). In guinea-pig atria, isoprenaline increases and propranalol decreases output of label following nerve stimulation. Although it is not known whether there are presynaptic $\beta$-adrenoceptors in the spleen the fact that labetalol is a competitive $\beta$-adrenoceptor antagonist (Farmer *et al.*, 1972) raises the possibility that it could act through this mechanism. Its relative lack of effect on transmitter overflow could be due to the antagonistic effects on $\alpha$-adrenoceptors tending to produce an increase, and $\beta$-adrenoceptor blockade tending to produce a decrease. We do not favour this explanation for several reasons. Firstly, the effects of $\beta$-adrenoceptor blockers are too small to explain the effects observed in the present experiments in the spleen. Secondly, in the spleen the addition of sotalol, a $\beta$-adrenoceptor antagonist without $\beta$ stimulant activity or membrane stabilizing effects (Barrett & Carter, 1970) produced a small decrease in transmitter overflow but did not affect the elevation produced by labetalol (Blakeley & Summers, unpublished observations). One would expect in these circumstances that following $\beta$-adrenoceptor blockade the presynaptic $\alpha$-adrenoceptor blocking effects of labetalol would be revealed, leading to a large increase in overflow. This did not occur. Another factor to be considered is the local anaesthetic property that labetalol is reported to have (Farmer *et al.*, 1972). A local anaesthetic effect could explain the abrupt decrease in overflow seen with high doses but does not appear to be important at lower dose levels since piperoxan was still able to elevate overflow considerably in the presence of these concentrations.

In conclusion we have provided evidence that labetalol is a preferential postsynaptic $\alpha$-adrenoceptor antagonist in the cat spleen. The moderate elevation of overflow which the drug produces in doses up to $10^{-4}$M is primarily due to inhibition of transmitter uptake rather than inhibition of presynaptic $\alpha$-adrenoceptors.

This work was supported by the Wood Boyd Fund of Glasgow University. The authors wish to thank Ms Eleanor Rafferty and Mr Gordon McCreaddie for excellent technical assistance; Dr John E. Pike of Upjohn, Kalamazoo for gifts of prostaglandin E$_1$; and Dr G.P. Levy of Allen and Hanburys Research Ltd. for gifts of labetalol.

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*(Received October 13, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 651–660

# A COMPARISON IN RABBIT ISOLATED HEARTS OF THE DYSRHYTHMOGENIC POTENTIAL OF AMITRIPTYLINE, MAPROTILINE AND MIANSERIN IN RELATION TO THEIR ABILITY TO BLOCK NORADRENALINE UPTAKE

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1 In isolated hearts of rabbits, perfusion with (–)-noradrenaline (0.0059 to 5.9 μM) resulted in chronotropic and inotropic responses and a shortening of the interval between peak atrial and peak ventricular tensions (the A–V contraction interval). No dysrhythmias developed but at higher concentrations (590 μM) 2 out of 7 hearts developed dysrhythmias (extrasystoles).

2 Perfusion with the antidepressants amitriptyline or maprotiline (4.8 μM) or mianserin (28.8 μM) reduced ventricular force, did not change heart rate and only amitriptyline reduced atrial force and lengthened the A–V contraction interval. At 4.8 μM mianserin produced only a marginal shortening of the A–V contraction interval.

3 At these concentrations no dysrhythmias developed but at higher concentrations (amitriptyline 8 μM, maprotiline 8 μM, mianserin 60 μM) all the agents produced dysrhythmias involving an interference with atrio-ventricular synchronization.

4 In the presence of mianserin (4.8 μM) perfusion with noradrenaline (0.0059 to 5.9 μM) shortened the A–V contraction interval and did not produce dysrhythmias. In the presence of amitriptyline or maprotiline (4.8 μM) or mianserin (28.8 μM) the A–V contraction interval generally lengthened and most hearts developed dysrhythmias (usually involving interference with atrio-ventricular synchronization).

5 [$^3$H]-(–)-Noradrenaline uptake in perfused rabbit hearts and in mouse isolated atria or vasa deferentia was inhibited by the antidepressants to a similar extent, amitriptyline being marginally most potent (molar potency taken as 1.0), maprotiline being less potent (1.5) and mianserin least potent (2.0).

6 It is concluded that of these three antidepressants, mianserin is least cardiotoxic in this preparation and that the ability of these antidepressants to predispose to noradrenaline-induced dysrhythmias is not related to blockade of noradrenaline uptake.

## Introduction

Administration of tricyclic antidepressants is known to be associated with the development of a variety of cardiac changes both in poisoning by these agents and after therapeutic use in man (Freeman, Mundy, Beattie & Ryan, 1969; Fouron & Chicoine, 1971; Williams & Sherter, 1971; Roberts, Mueller & Lauer, 1973). Concurrent use of sympathomimetic agents or procedures known to increase the levels of endogenous catecholamines appear to exacerbate the cardiotoxicity of the tricyclic antidepressants especially with regard to changes in the ECG. Thus, ECG changes have been observed in response to exercise in patients taking antidepressants (Kristiansen, 1961; Åsberg, Cronholm, Sjöqvist & Tuck, 1970) and infusions of sympathomimetics in healthy volunteers treated with imipramine tended to induce dysrhythmias (Boakes, Laurence, Teoh, Barar, Benedikter & Prichard, 1973). In animals, administration of tricyclic antidepressants in conjunction with noradrenaline has also been shown to produce dysrhythmias both *in vivo* (Elonen & Mattila, 1972; Elonen, Mattila & Saarnivaara, 1974) and *in vitro* (perfused rabbit heart) with either exogenous or endogenous noradrenaline (Barth & Muscholl, 1974; Barth, Manns & Muscholl, 1975). The mechanism by which these effects are produced is not clear but it has been suggested that the undoubted ability of antidepressants to block the uptake of noradrenaline will sensitize the heart to the dysrhythmogenic action of this catecholamine (Byck, 1976).

It was originally suggested by Barth & Muscholl in 1974 that the isolated perfused heart of the rabbit stimulated through associated sympathetic nerves might prove a satisfactory model on which to assess the relative cardiotoxicity of antidepressants. A technically simpler modification of this preparation involving administration of exogenous noradrenaline through the perfusate was later used successfully by Barth *et al.* (1975) in the assessment of the cardiotoxicity of several antidepressants and related drugs. They concluded that among tricyclic drugs the ability to inhibit noradrenaline uptake was related to the incidence of dysrhythmias evoked by noradrenaline but that the two parameters were not causally related. We have used this preparation with several modifications to assess the relative cardiotoxicity of the antidepressants amitriptyline, maprotiline (Mâitre, Waldmeier, Greengrass, Jaekel, Sedlacek & Delini-Stula, 1975; Reiss, Dubey, Fünfgeld, Imhof, Hürzeler, Matussek, Rajagopalan, Raschdorf & Schmid, 1975) and the tetracyclic antidepressant, mianserin (Itil, Polvan & Hsu, 1972; Fell, Quantok & van der Burg, 1973) with special reference to the ability of these compounds to inhibit the uptake of noradrenaline and to predispose to noradrenaline-induced dysrhythmias.

## Methods

### *Cardiotoxicity of the antidepressants*

Rabbits of either sex (1.5 to 4.0 kg; New Zealand or Californian White strains) were given heparin injection B.P. (2000 iu) through the ear vein 5 min before they were killed by a blow on the head. The heart was removed and placed in ice-cold physiological saline of the following composition, mM: NaCl 122, KCl 3.8, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 5.5, L-ascorbic acid 0.085; and aerated with 5% $CO_2$ in $O_2$. The heart was trimmed and mounted for perfusion through the coronary arteries by the method of Langendorff using a constant flow of 25 ml/min (Watson Marlow Flow inducer) at a temperature of 35.5°C. Atrial and ventricular tensions were recorded isometrically (Dynamometer UF1) from threads sewn into the heart tissue according to the 'transverse' method (Fozard & Muscholl, 1971; Barth & Muscholl, 1974). Heart rate was derived from the ventricular tension record with a Devices Instantaneous Rate-meter and perfusion pressure was monitored constantly. Ventricular tension, atrial tension, heart rate and perfusion pressure were displayed on Devices recorders (M2) and a Lan Oscilloscope (Lan-scope 419A) provided a visual monitor of atrial and ventricular tensions. The last two parameters were also displayed on a Mingograf 34 ink writer set to record at a fast speed on which periodic records were obtained and the time interval between the peak atrial and peak ventricular tensions determined (the A–V contraction interval).

Diastolic atrial and ventricular tensions were adjusted to 1.0 and 6.0 g respectively, the preparations were allowed to equilibrate for 15 min and the values of the above parameters were noted. Perfusion was then continued with physiological saline containing the drug under test for the rest of the experiment and after 15 min the values of the above parameters were again noted. Atrial and ventricular tensions were expressed as a percentage of the initial control values while changes in heart rate and A–V contraction interval were expressed in absolute terms. The preparations were then perfused for 5 min successively with each of several concentrations of (−)-noradrenaline (usually 0.0059, 0.019, 0.059, 0.19, 0.59, 1.9 and 5.9 μM) and any changes in the above parameters were noted 4 min after the start of perfusion. The concentrations of noradrenaline in the perfusion when any dysrhythmia was established was also noted. The experiments with the various drugs were performed in random order and each heart was used for one experiment only.

### *Uptake of $[^3H]$-(−)-noradrenaline in perfused rabbit heart*

Rabbit hearts were prepared as above, allowed to equilibrate for 15 min and were then perfused for a further 15 min with physiological saline containing the drug under test. $[^3H]$-(−)-noradrenaline was then added to the perfusion (0.059 μM; 3.33 nCi/ml) and the effluent from the heart was collected in successive 2 min periods for at least 20 minutes. A 1.0 ml sample of the effluent obtained at each collection period (and 3 x 1.0 ml samples of the un-perfused solution) was mixed with 10 ml of a dioxan-based scintillator (naphthalene 100 g, PPO 7 g, POPOP 0.5 g, methanol 50 ml, dioxan to 1000 ml) and counted for tritium in a Packard 3320 Liquid Scintillation Spectrometer. Correction for quench was made by an external standard channels ratio method. The amount of tritium in the effluent leaving the heart was expressed as a percentage of the amount entering the heart and hence the percentage removal of tritium from the perfusate was obtained and taken as an index of noradrenaline uptake.

### *Uptake of $[^3H]$-(−)-noradrenaline in mouse tissues*

Mature male mice (Tuck No. 1) were killed by a blow on the head; the heart and vasa deferentia were removed and placed in cold physiological saline (composition as above but L-ascorbic acid increased to 1.16 mM). Adherent tissue was carefully dissected away and the atria were separated from the ventricles. The atria and vasa deferentia were incubated in physiological saline (containing drugs when appropriate) for 15 min at 36°C. After this time the tissues were incubated in the presence of drug when appropriate for a further 15 min with [$^3$H]-(−)-noradrenaline (0.059 μM; 167 nCi/ml). The tissues were removed from the incubation solution, washed briefly in drug-free physiological saline at 4°C and then washed in 100 ml of physiological saline at 4°C for 15 min in the case of atria and 20 min in the case of vasa deferentia. The tissues were blotted dry on filter paper, weighed and combusted on tissue paper in a Packard Tri-Carb Oxidiser (305). The resulting solutions were counted for tritium as above and the tritium content of the tissue was expressed as ng noradrenaline base per g wet weight of tissue. The results were not corrected for recovery from the combustion process which was 90.8 ± 2.7% (mean ± s.e., $n = 10$) as determined by combustion of known amounts of [$^3$H]-(−)noradrenaline on tissue paper. In one group of tissues all the incubations were carried out at 4°C.

### *Drugs*

Amitriptyline hydrochloride (Laroxyl, Roche), L-ascorbic acid (BDH), maprotiline hydrochloride (Ludiomil, Ciba), mianserin hydrochloride (Bolvidon, Organon), (−)-noradrenaline bitartrate (Koch-Light) and [$^3$H](−)-noradrenaline (Radiochemical Centre, Amersham) were used. Stock solutions of noradrenaline were made up freshly each day in a solution of NaCl (153 mM), HCl (10 mM) and L-ascorbic acid (0.11 mM) and stored at 4°C. Dilutions in physiological saline were prepared immediately before use, the L-ascrobic acid being added immediately before the noradrenaline.

### *Statistical procedures*

Where appropriate all results are expressed as means ± standard error and in tests for statistical significance Student's $t$ test was used. Regression analysis was carried out according to Snedecor & Cochran (1967).

## Results

### *Effects of the antidepressants amitriptyline, maprotiline and mianserin alone*

At the end of the initial equilibration period the values for the recorded parameters in a group of 6 control hearts were: (mean ± s.e.) ventricular force 9.8 ± 0.8 g, atrial force 0.78 ± 0.13 g, heart rate 125.0 ± 2.2 beats/min, perfusion pressure 31.8 ± 2.1 mmHg (4.23 ± 0.28 kPa) and A–V contraction interval (the interval between peak atrial and peak ventricular force) 168.6 ± 10.6 milliseconds. The groups of hearts later to be perfused with drug-containing solutions showed no statistically significant differences from the above values.

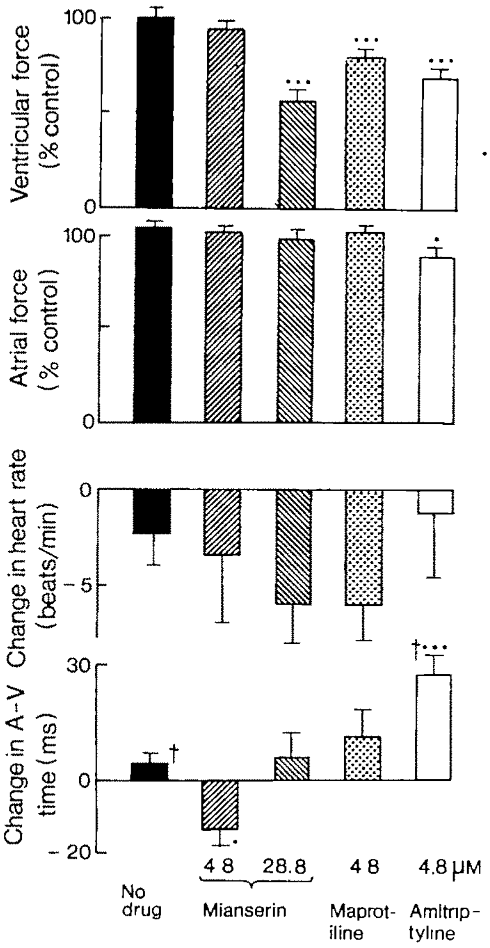

**Figure 1** The changes produced in the performance of isolated perfused hearts of rabbits (6 per group) by 15 min perfusion with mianserin (4.8 and 28.8 μM), maprotiline (4.8 μM), amitriptyline (4.8 μM) or physiological saline alone (no drug). Atrial and ventricular force are expressed as a percentage of the initial values obtained immediately before the start of this perfusion. The changes in heart rate and in A–V contraction interval (the interval between peak atrial and peak ventricular tensions) are given in absolute terms. The error bars represent ± s.e. mean and the asterisks indicate statistically significant differences from perfusion with physiological saline alone (* $P < 0.05$ but $> 0.02$; *** $P < 0.01$). †, only 5 values contributed to this observation in this group.

After a further 15 min perfusion with physiological saline alone, control hearts showed no significant changes in these parameters (Figure 1). Perfusion with amitriptyline (4.8 μM) reduced ventricular force and atrial force and lengthened the A–V contraction

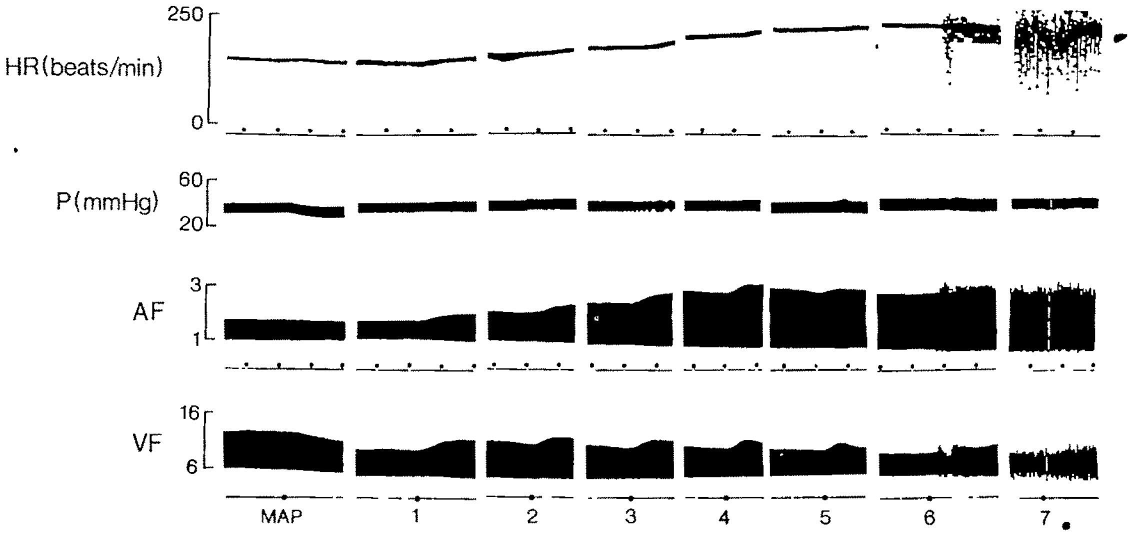

**Figure 2** Records of heart rate (HR, beats/min), perfusion pressure (P, mmHg), atrial force (AF, g) and ventricular force (VF, g) from an isolated perfused heart of rabbit which was exposed to maprotiline (MAP, 4.0 μM) for 15 min and then perfused for 5 min successively with each of various concentrations of (—)-noradrenaline (1–7, 0.0059, 0.019, 0.059, 0.19, 0.59, 1.9 and 5.9 μM respectively). Note the initial chronotropic and inotropic responses to noradrenaline and the dysrhythmia developing after 30 s perfusion with noradrenaline at a concentration of 1.9 μM. Time marker 60 seconds.

interval but did not change heart rate. Maprotiline (4.8 μM) also reduced ventricular force but did not change the other parameters in a statistically significant manner. Mianserin (4.8 μM) had no significant effect on ventricular force, atrial force or heart rate but produced a small but statistically significant shortening of the A–V contraction interval. At a higher concentration (28.8 μM), ventricular force was reduced significantly and a marginal increase in the A–V contraction interval was observed though this latter effect was not statistically significant. All these changes are presented in histogram form in Figure 1.

In none of these experiments was cardiac dysrhythmia observed with the concentrations of the drugs under test. In single experiments however it was found that all three agents were capable of producing cardiac dysrhythmias. When hearts were exposed to step-wise increasing concentrations of the antidepressants (2, 4, 8, 20, 40 and 60 μM) a progressive lengthening of the A–V contraction interval was noted which culminated in a dysrhythmia (amitriptyline 8 μM; maprotiline 8 μM; mianserin 60 μM).

### *Effects of noradrenaline in the presence and absence of the antidepressants*

When perfused with noradrenaline at concentrations of up to 5.9 μM (the highest used routinely) none of the control hearts, developed dysrhythmias but in the presence of the antidepressants, dysrhythmias often developed during perfusion with low concentrations of noradrenaline and, once developed, were maintained for the remainder of the perfusion period. A typical record from an experiment is shown in Figure 2. Note the initial inotropic and chronotropic responses to low concentrations of noradrenaline and the maintained dysrhythmias developing after 30 s perfusion with noradrenaline at a concentration of 1.9 μM. Six hearts were tested in this way at each of the concentrations of the drugs examined and Figure 3 presents the numbers of hearts which showed dysrhythmias at the various concentrations of noradrenaline used. Control hearts and those perfused with mianserin (4.8 μM) did not develop noradrenaline-induced dysrhythmias, neither did those perfused with low concentrations of amitriptyline (1.0 μM, 3 expts.; 2.0 μM, 1 expt.) but at 4.8 μM, 2 of the hearts developed a dysrhythmia at the lowest concentration of noradrenaline (0.0059 μM) and all 6 were eventually affected. Maprotiline appeared to be marginally less effective in predisposing to noradrenaline-induced dysrhythmias since only 1 of the 6 hearts became dysrhythmic with noradrenaline concentrations up to 0.059 μM, but at higher noradrenaline concentrations all were eventually affected. With mianserin (28.8 μM) 4 of the hearts became dysrhythmic eventually but the remaining 2 did not develop a dysrhythmia even at the highest concentration of noradrenaline used routinely (5.9 μM).

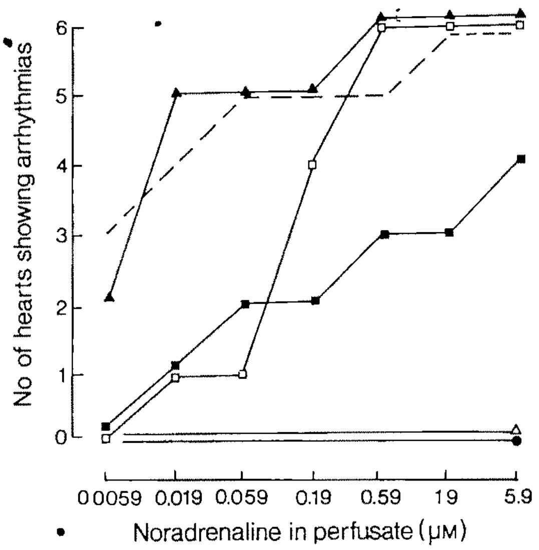

**Figure 3** The number (out of 6) of rabbit isolated perfused hearts exhibiting dysrhythmias during perfusion with various concentrations of noradrenaline alone (control, ●) or in the presence of amitriptyline (▲, 4.8 μM), maprotiline (□, 4.8 μM) or mianserin (△, 4.8 μM; ■, 28.8 μM). For clarity some points have been marginally off-set from their correct values on the abscissa scale and all points have been omitted from the control and mianserin (4.8 μM) lines. The broken line represents the results obtained for amitriptyline (4.8 μM) by Barth *et al.* (1975).

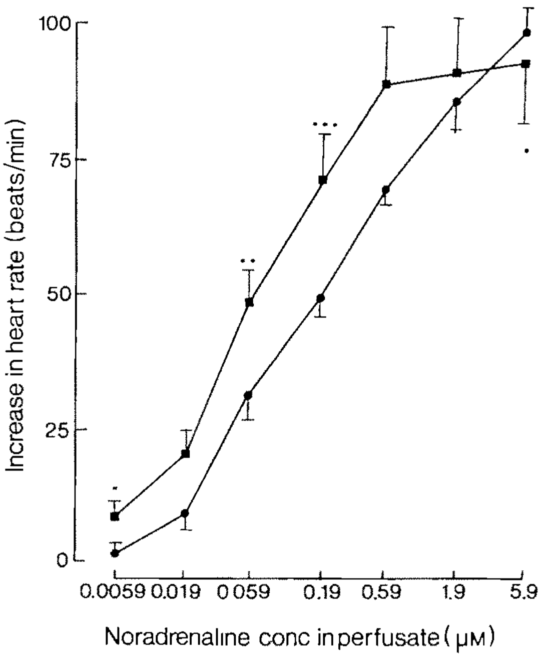

**Figure 4** Increases in heart rate (beats/min) in rabbit isolated hearts in response to perfusion with various concentrations of (–)-noradrenaline alone (●, $n=6$) or in the presence of mianserin (■, 4.8 μM; $n=6$). The resting heart rates in the control group (125.0 ± 2.2 beats/min) and the group perfused with mianserin (121.3 ± 8.7 beats/min) were not significantly different ($P>0.8$). Mean values are shown; bars indicate s.e. means. The asterisks indicate statistically significant differences from control responses at corresponding noradrenaline concentrations: * $P<0.05$ but $>0.02$; ** $P<0.02$ but $>0.01$; *** $P<0.01$.

In addition to producing dysrhythmias, noradrenaline perfusions in the presence of the antidepressants also produced inotropic responses. These could not readily be quantitated since dysrhythmias occurred in many cases and because steady-state responses were rarely seen. The chronotropic response however usually did reach a steady-state and it can be seen that in the case of mianserin (4.8 μM; the only solution tested that did not provoke dysrhythmias in the presence of noradrenaline) there was a potentiation of the chronotropic response to noradrenaline in comparison with control tissues (Figure 4).

Noradrenaline perfusions also affected the A–V contraction interval and the values obtained for this parameter in each heart during perfusion with the various concentrations of noradrenaline are shown in Figure 5. No attempt has been made to calculate means but it is clear that in control hearts noradrenaline produced a shortening of the A–V contraction interval.

In contrast to this effect, in the presence of amitriptyline (4.8 μM) or maprotiline (4.8 μM), a lengthening of the A–V contraction interval was observed. In the presence of mianserin (4.8 μM) the normal action of noradrenaline (to shorten the A–V contraction interval) was still seen, although possibly to a lesser extent, but at 28.8 μM (as with amitriptyline and maprotiline at 4.8 μM) a progressive lengthening of the A–V contraction interval was usually seen, terminating in a dysrhythmia. In 2 hearts the A–V contraction interval was not markedly lengthened and neither of these hearts developed dysrhythmias.

The dysrhythmias seen in response to noradrenaline in the presence of the antidepressants were somewhat variable in nature but usually involved an apparent interference with atrio-ventricular conduction; missed ventricular beats were common as was atrio-ventricular dissociation. In the absence of the antidepressants even at extremely high noradrenaline concentrations (590 μM) only 2 out of 7 hearts showed dysrhythmias. In both cases the dysrhythmias involved extrasystoles and in neither case was any lengthening of the A–V contraction interval seen.

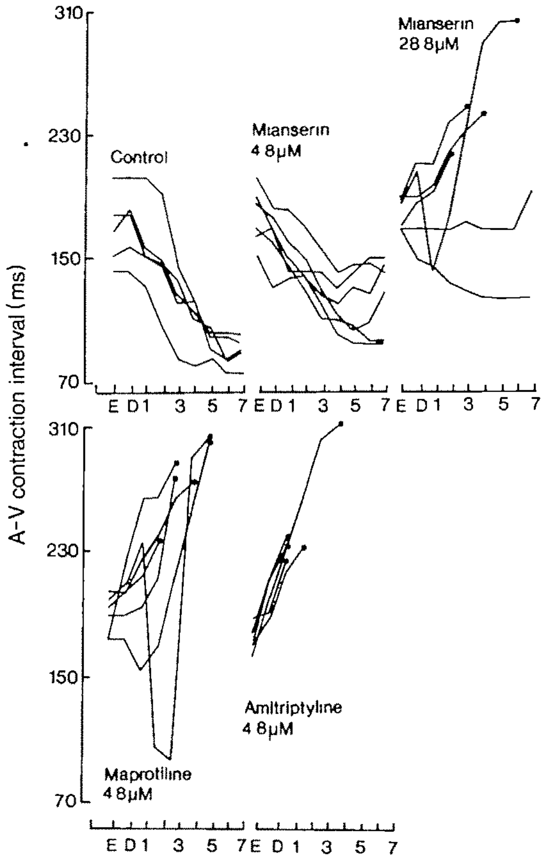

**Figure 5** Effect of noradrenaline on the A–V contraction interval (interval between peak atrial and peak ventricular tensions; ms) in the presence and absence of the antidepressants. Each line represents an isolated perfused heart of a rabbit and measurements were taken: E, at the end of an equilibration period; D, after 15 min perfusion with a drug when appropriate; 1–7, during perfusion with (–)-noradrenaline (0.0059, 0.019, 0.059, 0.19, 0.59, 1.9 and 5.9 μM respectively). Lines ending in closed circles indicate that a dysrhythmia developed before the next measurement of the A–V contraction interval could be made.

### *Effects of the antidepressants on [$^3$H]-(–)-noradrenaline uptake*

Measurement of the $^3$H-content of the effluent from hearts perfused with [$^3$H]-(–)-noradrenaline (0.059 μM) showed that the amounts of tritium apparently 'removed' from the perfusate were very high initially as the H$^3$-content of the perfusate was diluted by mixing with the physiological saline already in the 'dead space' of the apparatus (28 ml). However, in both control and treated hearts a plateau was quickly reached which usually remained constant for the remainder of the experiment (20 minutes). In a single control experiment the amount of noradrenaline in the effluent from the heart was also measured fluorimetrically by a modified trihydroxyindole method applied directly to the perfusate. There was general agreement between the fluorimetric and isotopic methods though the fluorimetric method consistently indicated a greater removal of noradrenaline by the heart than did the isotopic method (Figure 6).

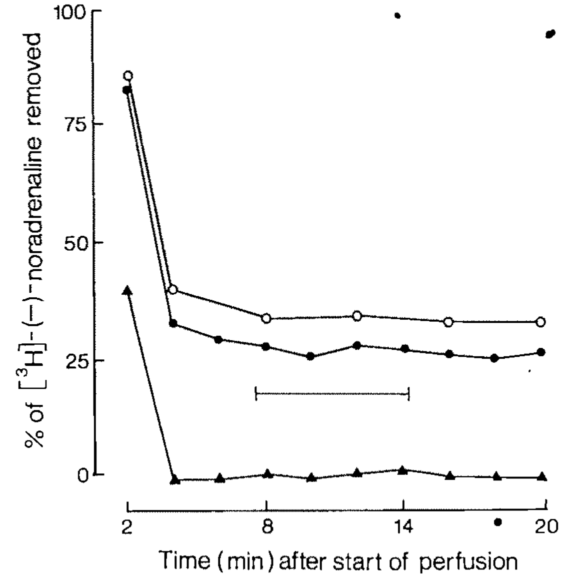

**Figure 6** The percentage of [$^3$H]-(–)-noradrenaline removed from the perfusate by a single passage through an isolated perfused heart of rabbit. Assessment of the noradrenaline content of the effluent was made either fluorimetrically (open symbols) or isotopically (closed symbols) in control hearts (○ ●) or in the presence of mianserin (▲, 4.8 μM). The ordinate scale shows the time (min) from the start of the perfusion with [$^3$H]-(–)-noradrenaline. To calculate the percentage removal by each heart (see Figure 7) the average was taken of the points included by the solid bar.

The percentage removal of noradrenaline for each control or treated heart was calculated as the average removal over 4 samples (each involving a 2 min collection period) obtained between 6 and 14 min after starting the perfusion with [$^3$H]-(–)-noradrenaline. The percentage removed by control hearts (28.4 ± 0.7%; $n = 7$) was taken as 100% uptake and the amounts removed by treated hearts were expressed as a percentage of this uptake.

In single experiments (at 4.8 μM; the concentration used in investigation of the dysrhythmogenic potential of the agents) all three antidepressants effectively eliminated uptake of [$^3$H]-(–)-noradrenaline by the rabbit heart and the relationship between the concentration of antidepressant in the perfusate and the uptake of noradrenaline (as a percentage of control) is shown in Figure 7. It may appear that the lines are not parallel but the errors are such that neither the slope of the maprotiline line $(-72.3 \pm 8.0, n=8)$ nor the slope of the mianserin line $(-45.1 \pm 5.1, n=11)$ differ significantly $(P>0.05)$, from the slope of the line determined for amitriptyline $(-64.1 \pm 8.8, n=9)$. Calculation from the regression equations of the concentration of the antidepressants required to produce 50% blockade of the uptake of noradrenaline yields the values shown in Table 1. Isolated atria and vasa deferentia of mice incubated with [$^3$H]-(−)-noradrenaline in the absence of other drugs retained some $57.4 \pm 4.3$ $(n=8)$ and $45.2 \pm 1.7$ $(n=20)$ ng noradrenaline base per g wet weight of tissue respectively. In the presence of cocaine (30 μM) the amount of noradrenaline retained was reduced to 15% of control in both cases while in tissues incubated at 4°C only 3% and 5% of normal control uptake occurred in atria and vasa deferentia respectively. The relationship between the uptake of noradrenaline and the concentration of antidepressant in the incubation fluid is shown in Figure 8 and calculation of the concentration of antidepressant required to produce 50% blockade of the uptake of noradrenaline yields the values shown in Table 1.

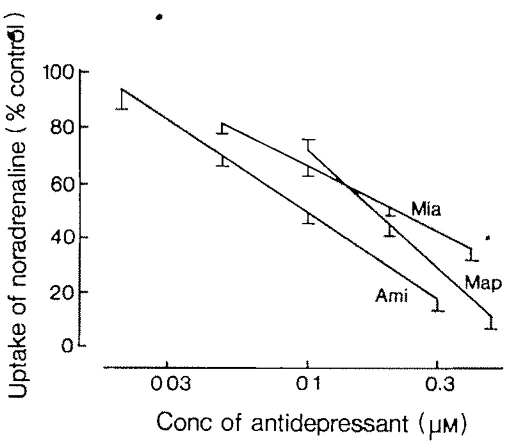

**Figure 7** The relationship in rabbit isolated hearts between the concentration of antidepressant in the perfusate and the uptake of [$^3$H]-(−)-noradrenaline (expressed as a percentage of the control uptake which was $28.4 \pm 0.7\%$ $(n=7)$ of the noradrenaline (0.059 μM) delivered to the heart). The lines shown (mianserin (Mia), $n=11$; maprotiline (Map), $n=8$; amitriptyline (Ami), $n=9$) are regression lines calculated from results obtained in several experiments at various concentrations. The error bars show the standard error of regression calculated at the concentrations at which the experiments were performed.

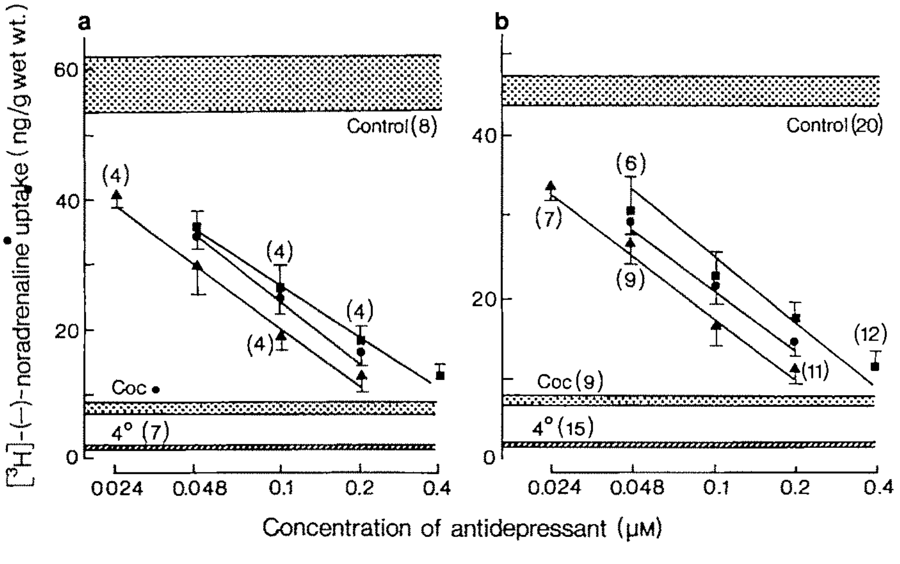

**Figure 8** The amounts of [$^3$H]-(−)-noradrenaline (ng per g wet weight of tissue) taken up by (a) isolated atria and (b) vasa deferentia of mice after incubation for 15 min with [$^3$H]-(−)-noradrenaline in the presence of various concentrations of mianserin (■), maprotiline (●) or amitriptyline (▲). Points are mean values; bars show s.e. means. The lines are calculated regression lines based on the individual results from which the means were derived. Also shown is the uptake in the absence of drugs (control), in tissues incubated in the presence of cocaine (Coc, 30 μM) or at 4°C. In each case the upper and lower limits of the shaded areas show the mean plus and minus one s.e. respectively. Five tissues contributed to each group in the case of the atria (10 in the case of vas deferens) except where indicated (figures in parentheses).

## Discussion

The use of the isolated perfused heart of the rabbit in investigations of antidepressant cardiotoxicity was established by Barth *et al.* (1975) who investigated the effects of a number of antidepressants in this preparation and the results described here for amitriptyline are in agreement with those obtained by these previous workers. We also found that at 4.8 μM, amitriptyline reduced the force of atrial and ventricular systole but had no effect on heart rate and that perfusion with low concentrations of noradrenaline in the presence of amitriptyline gave rise to dysrhythmias.

At equimolar concentrations (4.8 μM) amitriptyline reduced the force of ventricular systole to a greater extent than did maprotiline while mianserin produced no significant change. At 28.8 μM the effects of mianserin on ventricular force were slightly greater than those of amitriptyline and it would appear therefore that mianserin is less potent than either amitriptyline or maprotiline in this respect.

The relative ability of the three antidepressants to predispose to noradrenaline-induced dysrhythmias is more difficult to quantitate exactly because of the nature of the experimental design. At equimolar concentrations (4.8 μM) amitriptyline appeared more likely to precipitate dysrhythmias at low concentrations of noradrenaline than did maprotiline and no dysrhythmias developed with mianserin. Even when the concentration of mianserin was increased 6-fold only 4 out of the 6 hearts developed dysrhythmias when perfused with noradrenaline. This is not because mianserin prevents the actions of noradrenaline on the heart as inotropic responses were still seen and the chronotropic response was significantly potentiated compared to control tissues. This latter observation suggests the possibility that mianserin, in common with other antidepressants, can interfere with the uptake of noradrenaline in the periphery as it does in rat brain synaptosomes (Raiteri, Angelini & Bertollini, 1976).

This possibility was confirmed in the experiments investigating the uptake of [$^3$H]-(−)-noradrenaline in various tissues. Throughout this work it has been assumed that the $^3$H-content of various solutions and tissues can be taken as an index of their noradrenaline content or uptake respectively. Although the percentage of noradrenaline removed from the perfusate was consistently higher when assessed fluorimetrically than when assessed by the isotopic method, correspondence was generally good. A higher result might in any case be expected since any noradrenaline metabolized will show as a loss of noradrenaline (i.e. uptake) in the fluorimetric method while this is not so in the isotopic method. With regard to uptake of noradrenaline in mouse tissues we have no evidence to suggest that the tritium in the tissue is present as noradrenaline. However, given the selectivity of the uptake process and the fact that [$^3$H]-(−)-noradrenaline is chemically stable in the incubation solution the $^3$H-content of the tissue must have derived from uptake of noradrenaline and the form in which it exists in the tissue after uptake is irrelevant in this context.

In perfused rabbit hearts the concentration of amitriptyline required to produce 50% inhibition of [$^3$H]-(−)-noradrenaline uptake (100 nM) agrees well with the value found by Barth and his co-workers (128 nM), who used a fluorimetric assay. The uptake blocking potencies of the compounds in the three preparations show reasonable agreement; in each case amitriptyline is the most potent, maprotiline marginally less so and mianserin approximately 2-fold less potent than amitriptyline. At 4.8 μM all the compounds produced complete blockade of the uptake of [$^3$H]-(−)-noradrenaline in perfused hearts although their relative liability to predispose to noradrenaline-induced arrhythmias was very different. This would suggest that predisposition to noradrenaline-induced dysrhythmias is not directly associated with an ability to block uptake of noradrenaline. This suggestion is supported by several other aspects of the results described here.

Firstly, noradrenaline by itself, even in concentrations some 10,000-fold greater than those needed to produce dysrhythmias in the presence of amitriptyline or maprotiline, only produced dysrhythmias in 2 out of 7 hearts. Complete blockade of noradrenaline uptake by minaserin produced only a 4-fold potentiation in the chronotropic response to noradrenaline and in other tissues complete blockade

**Table 1** Concentrations (nM) of amitriptyline, maprotiline and mianserin required to produce 50% inhibition of the uptake of [$^3$H]-(−)-noradrenaline in isolated organs of rabbit and mouse

| *Drug* | *Rabbit heart* | *Mouse atria* | *Mouse vas deferens* |
|---|---|---|---|
| Amitriptyline | 100 | 53.6 | 64.9 |
| Maprotiline | 152 (1.52) | 75.5 (1.41) | 89.5 (1.38) |
| Mianserin | 201 (2.01) | 88.9 (1.66) | 131.0 (2.02) |

The figures in parentheses show the equi-active molar potency ratios relative to amitriptyline.

of uptake rarely produces more than a 30 to 50-fold potentiation in the response (Trendelenberg, 1972). If the predisposition to noradrenaline-induced dysrhythmias were solely due to blockade of noradrenaline uptake it would be expected that a 50 to 100-fold increase in the concentration of noradrenaline perfused through the heart would, by itself, produce dysrhythmias in all hearts but no such effect was seen.

Secondly, in the two hearts which did become dysrhythmic in the presence of high concentrations of noradrenaline, the dysrhythmias involved the production of extrasystoles and there was no evidence of any interference with atrio-ventricular synchronization or lengthening of the A–V contraction interval. In contrast, in hearts exposed to noradrenaline as well as an antidepressant, extrasystoles were rarely seen. Usually the dysrhythmias involved an interference with atrio-ventricular conduction. It appears therefore that the type of dysrhythmia induced by noradrenaline alone is different from that seen with noradrenaline in the presence of an antidepressant, a feature that would not be expected if the dysrhythmia were simply due to potentiation of the normal effects of noradrenaline. In this context it is interesting to note that the dysrhythmias induced by antidepressants in conscious rabbits were also characterized by atrio-ventricular conduction defects (Elonen *et al.*, 1974) and it may be conceptually more useful to think of noradrenaline predisposing to an antidepressant type of dysrhythmia rather than antidepressants predisposing to noradrenaline dysrhythmias.

The third factor which suggests that blockade of noradrenaline uptake is not solely responsible for the precipitation of dysrhythmias comes from a consideration of the effects on A–V contraction interval. The use of this measurement is fraught with difficulties since it represents the time interval between two mechanical events which may, through alterations in rise time for example, bring about a change in the measured A–V contraction interval which bears little relationship to changes in electrical conduction that may have taken place. Nevertheless noradrenaline shortened the A–V contraction interval in normal hearts and is known to speed atrio-ventricular conduction. A–V contraction interval was increased by amitriptyline alone and this agent is known to increase the P–Q interval in the ECG of the conscious rabbit (Elonen *et al.*, 1974).

Noradrenaline shortened the A–V contraction interval in normal hearts and also in the presence of mianserin (4.8 μM) though the effect was possibly less marked. In contrast, with both maprotiline and amitriptyline a progressive lengthening of the A–V contraction interval was seen as more concentrated solutions of noradrenaline were applied. Indeed, so long did the A–V contraction interval become in some hearts that the atrial contraction immediately preceding a ventricular contraction did not in fact trigger that contraction, but the next ventricular event. Thus a very long A–V contraction interval could easily be mistaken for a very short A–V contraction interval unless the changes are constantly monitored. In the presence of mianserin (28.8 μM) 4 hearts showed a lengthening of the A–V contraction interval and developed dysrhythmias while the remaining 2 did not develop dysrhythmias or show any marked lengthening of the A–V contraction interval.

It seems likely therefore that the dysrhythmogenic action of noradrenaline in the presence of the antidepressants cannot be due simply to a blockade of noradrenaline uptake. The primary cause in this preparation would appear to be the ability of these compounds to interfere with the normal effects of noradrenaline on the A–V contraction interval which may be related to the intrinsic ability of the molecular structures involved to produce the effect. The presence of an additional ability to block the uptake of noradrenaline would exacerbate this action as the effective concentration of noradrenaline would be raised but this is not the primary cause of the ability of these compounds to predispose to noradrenaline-induced dysrhythmias.

Any extrapolation of these results to the clinical situation in man must be done with extreme caution. Quite apart from any species variation and the fact that the experiments described above do not involve chronic administration, the effects of vagal and other influences on the heart *in vivo* may grossly modify the effects observed. Furthermore, plasma levels of amitriptyline may approach 300 ng/ml in clinical use (Braithwaite & Widdop, 1971; Jørgensen, 1975) and may be much higher in poisoning but allowing for 95% plasma binding (Borgå, Azarnoff, Forshell & Sjövist, 1969) this represents a free drug concentration of 50 nM, some 100-fold below the concentration used to predispose to dysrhythmias in these experiments.

We would like to express our thanks to Roche Products Ltd. for a gift of amitriptyline, to Ciba Laboratories Ltd. for a gift of maprotiline, to Organon International, Holland, for financial support and to Anne Wells and Mr K. Heald for technical assistance.

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*(Received November 2, 1976.)*

*Br. J. Pharmac.* (1977), **59**, 661–662

# ASPIRIN AT THERAPEUTIC CONCENTRATIONS DOES NOT AFFECT 5-HYDROXYTRYPTAMINE UPTAKE BY PLATELETS

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Aspirin at therapeutic concentrations does not inhibit the uptake of 5-hydroxytryptamine (5-HT) by blood platelets nor induce release of 5-HT from platelets, although platelet aggregation responses to collagen and arachidonic acid (which are dependent on platelet prostaglandin synthesis) are abolished. This does not support an earlier claim that aspirin's effects on platelet function include inhibition of 5-HT transport.

**Introduction** Aspirin at plasma concentrations of 0.1–1 mM inhibits prostaglandin-dependent platelet aggregation and secretion (for review, see Smith & Silver, 1977). Rendu (1976) showed that 1.1 mM aspirin inhibited the uptake of 5-hydroxytryptamine (5-HT) by human platelets, and suggested that this action might contribute significantly to the spectrum of aspirin's inhibitory effects on platelets. A subsequent correction (Erratum: *Br. J. Pharmac.* (1976), **58** (1)) indicated that an aspirin concentration of 110 mM had in fact been used in these experiments, but this correction did not explain how a concentration of 110 mM aspirin (which greatly exceeds the drug's aqueous solubility) was achieved in a physiological medium, and therefore more methodological details of these experiments are needed to evaluate the results adequately. In addition, the question originally addressed by Rendu (1976) remained unanswered—does aspirin at therapeutic concentrations affect 5-HT uptake by platelets? We therefore measured the uptake of 5-HT by platelets in plasma samples with up to 1 mM aspirin added, and in samples from volunteers who had ingested aspirin. The potency of aspirin against platelet responses that are dependent on prostaglandin synthesis was tested by measuring aggregation induced by collagen and arachidonic acid in replicate samples.

**Methods** Human citrated platelet rich plasma (PRP) was prepared by differential centrifugation of blood from volunteers who had taken no medication for at least 10 days. Uptake of 5-HT was measured as described by Drummond & Gordon (1976). Briefly, 0.1 ml samples of PRP were incubated with 5-hydroxy-[G-$^3$H]-tryptamine creatinine sulphate (0.5 Ci/mmol; The Radiochemical Centre, Amersham), final concentration 1 μM, at 37°C for 2 min except where otherwise stated. Uptake was terminated by addition of 0.5 ml ice cold iso-osmotic saline containing 0.4% (w/v) disodium edetate (EDTA) and centrifuging immediately (14,700 *g*; 30 seconds). The cell pellet was washed with 1 ml iso-osmotic saline and the uptake of 5-HT determined by liquid scintillation counting of the radioactivity in the pellet. A 5-HT concentration of 1 μM was chosen because this is the apparent $K_m$ value for 5-HT uptake by human platelets under these conditions (Gordon & Olverman, 1976).

Platelet aggregation was measured photometrically (Born, 1962) in 0.1 ml samples of PRP (Gordon & Drummond, 1974). Agents used to induce platelet aggregation were fibrillar collagen (10 μg/ml) and arachidonic acid (1 mM). Both of these agents stimulate platelets by activating prostaglandin synthesis.

For studies *in vitro* aspirin (final concentrations 5–1000 μM) was preincubated in PRP at 37°C for 10 minutes. For studies *ex vivo*, PRP was prepared from volunteers immediately before and 2 h after ingestion of 900 mg aspirin. The platelet count in each sample was adjusted to $3.5 \times 10^8$ cells/ml.

**Results** Uptake of 5-HT 2 h after ingestion of aspirin was unaltered (Figure 1a) although platelet aggregation responses to collagen and arachidonic acid were abolished (Figure 1b). When tested *in vitro*, aspirin in concentrations of 25–1000 μM progressively inhibited aggregation induced by collagen and arachidonic acid, but did not affect 5-HT uptake (Figure 1c).

Since Rendu (1976) had shown that the effect of aspirin on 5-HT transport was associated with 5-HT being released from the platelets, we investigated the effects of 1.1 mM aspirin on platelets preloaded with [$^3$H]-5-HT. Samples of PRP were incubated for 30 min at 37°C with 1 μM [$^3$H]-5-HT. The amount of

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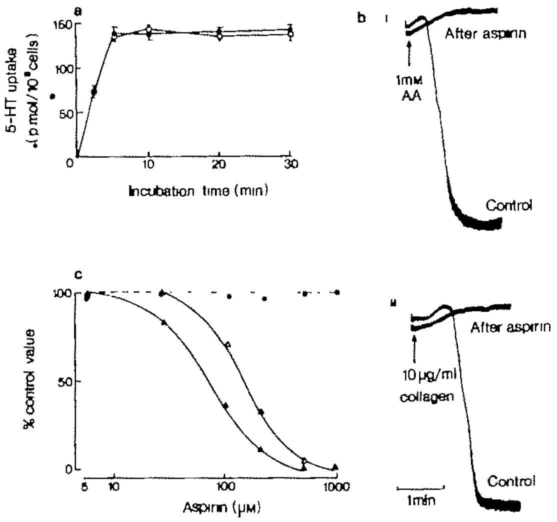

**Figure 1** Effect of aspirin on the uptake of 5-hydroxytryptamine (5-HT) by human platelets and on platelet aggregation induced by collagen (10 μg/ml) and arachidonic acid (1 mM). (a) 5-HT uptake measured before (●) and 2 h after (○) ingestion of 900 mg aspirin. Points shown are means, vertical bars show s.e. mean, $n=6$. Samples of PRP were incubated at 37°C with 1 μM [$^3$H]-5-HT, and uptake was determined as described in the text. (b) Platelet aggregation responses to (i) 1 mM arachidonic acid (AA) and (ii) to 10 μg/ml collagen, measured before and 2 h after ingestion of 900 mg aspirin. Aggregating agents were added at the arrows, and responses measured photometrically (see text). (c) 5-HT uptake and platelet aggregation responses measured after 10 min preincubation with aspirin (5–1000 μM). Replicate samples of PRP were taken for determination of 5-HT uptake (●) as described in the text, and for measurement of aggregation responses to 10 μg/ml collagen (▲) and to 1 mM arachidonic acid (△). All measurements are expressed as percentage of values obtained in control samples containing an equivalent volume of iso-osmotic saline. Points shown are means of triplicate determinations.

[$^3$H]-5-HT transported was (mean ± s.e.) $143 \pm 3$ pmol/$10^8$ cells ($n=6$). Addition of 1.1 mM aspirin did not release any [$^3$H]-5-HT: 30 min after adding aspirin the platelet content of [$^3$H]-5-HT was $146 \pm 4$ pmol/$10^8$ cells, and in matched control samples the platelet content of [$^3$H]-5-HT, 30 min after adding an equivalent volume of iso-osmotic saline, was $144 \pm 3$ pmol/$10^8$ cells.

**Discussion** Therapeutic doses of aspirin result in plasma levels of the drug between 0.1 and 0.5 mM, but concentrations above 1 mM are not achieved even after high doses. Results from many laboratories (including our own) have shown that aspirin at therapeutic concentrations can abolish platelet responses that are dependent on prostaglandin biosynthesis, but the results of our present study clearly demonstrate that biologically significant concentrations of aspirin do not inhibit 5-HT uptake or stimulate 5-HT secretion.

This study was supported by grants from the Medical Research Council and from the Arthritis and Rheumatism Council.

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*(Received January 6, 1977.)*

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VOLUME 59, 1977

LONDON
MACMILLAN JOURNALS LIMITED
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